# Sialoglycoproteins of Human Mammary Cells: Partial Characterization of Sialoglycopeptides†

E. V. Chandrasekaran and E. A. Davidson*

ABSTRACT: Sialoglycopeptides were isolated and fractionated from cultured human mammary cell lines (one control line, HBL-100, and two cancer lines, MDA-MB-231 and MCF-7) into three groups: neutral, less anionic, and more anionic. Cells were cultured with [$^3$H]glucosamine, [$^3$H]glucosamine and [$^{14}$C]galactose, or [$^{14}$C]glucosamine and [$^3$H]galactose, following which spent-medium and cell-associated components were analyzed. Exhaustive Pronase digestion and removal of glycosaminoglycans with cetylpyridinium chloride were followed by diethylaminoethylcellulose and wheat germ agglutinin (WGA)–Sepharose chromatography. The cancer lines produced 3–5 times greater amounts of the more anionic glycopeptides compared to the control line. Within this fraction, WGA-bound material was predominant in the cancer lines. Alkaline borohydride treatment of the WGA-binding fraction from both cancer lines gave rise to a major elimination product not observed with comparable material from HBL-100 cells. Analysis of the asialosaccharide chain gave the structure Gal$\overset{\beta}{\leftarrow}$GlcNAc$\overset{\beta}{\leftarrow}$Gal→GalNAcOH. The structural requirements for the binding of the glycopeptides to WGA were analyzed. The WGA-binding capacity is dependent on both molecular size and sialic acid content. In addition, the saccharide moiety of the WGA-binding glycopeptides is purely O-glycosidically linked whereas that from the WGA-nonbinding material is mainly N-glycosidic. The WGA-binding glycopeptides contain clustered oligosaccharide chains having the sequence NeuNAc$\overset{\alpha}{\rightarrow}$Gal$\overset{\beta}{\leftarrow}$GlcNAc$\overset{\beta}{\leftarrow}$Gal→GalNAc→Ser(Thr).

Several investigations have led to the finding that transformed cells differ from normal cells in sialic acid content, sialoglycoproteins, and sialoglycolipids (Grimes, 1973; Warren et al., 1972; Mora et al., 1969; Ohta et al., 1968; Hakomori, 1970). Bosmann & Hall (1974) have observed an increased level of glycoprotein–sialyl transferase activity in human malignant neoplastic tissues. The presence of a highly sialylated mucin-type glycoprotein in mouse melanoma cells and not in normal melanocytes has been demonstrated in our laboratory (Bhavanandan et al., 1977). Recently we reported that two different human breast cell lines differ from a normal human mammary cell line in the synthesis of glycosaminoglycans (Chandrasekaran & Davidson, 1979). The nature of the sialoglycopeptides produced by these cell lines is the subject of this paper. We describe an elevated production of mucin-type sialoglycoproteins by both breast cancer cell lines as compared to the control line, the structure of the O-glycosidic oligosaccharide chain from the cancer lines, and a preliminary characterization of the cancer-related mucin-type sialoglycopeptides.

## Experimental Procedures

*Materials.* Flow Laboratories (Rockville, MD) supplied fetal calf serum, and Grand Island Biological Co. (Grand Island, NY) supplied the culture medium components. New England Nuclear (Boston, MA) provided [6-$^3$H]glucosamine hydrochloride, [6-$^3$H]galactose, [1-$^{14}$C]glucosamine hydrochloride, and [1-$^{14}$C]galactose. Highly purified $\beta$-galactosidase from jack bean was kindly provided by Dr. Y. T. Li of Tulane University, New Orleans, LA. DEAE-cellulose (fine mesh), $\beta$-galactosidase (EC 3.2.1.23) (*Aspergillus niger*), $\beta$-*N*-acetylglucosaminidase (EC 3.2.1.30) (jack bean), and $\alpha$-galactosidase (EC 3.2.1.22) (green coffee bean) were purchased from Sigma Chemical Co. (St. Louis, MO); $\beta$-galactosidase and $\beta$-*N*-acetylhexosaminidase from *Diplococcus pneumoniae* were prepared in this laboratory.

Culture conditions, isotopic labeling procedures, and processing of cells or medium for glycopeptide isolation were as described in earlier reports (Bhavanandan et al., 1977; Chandrasekaran & Davidson, 1979). Cells were routinely exposed to isotopic precursor at confluence. In a single experiment, subconfluent logarithmically growing cells yielded products which were similar in chromatographic behavior and amount. The glycopeptides after Pronase digestion represented better than 90% of the cell-associated macromolecular radioactivity. The nature of the nonsolubilized material was not studied further. The cetylpyridinium chloride (CPC)[1] supernatant obtained after the removal of precipitated GAGs was treated with 2% KCNS to precipitate the CPC and centrifuged, and the supernatant was dialyzed first against water for 24 h at 37 °C and then for an additional 48 h at 4 °C. The dialysate was concentrated to dryness by evaporation, in vacuo, dissolved in a small volume of water, and then chromatographed.

*DEAE-cellulose Chromatography.* The glycopeptide material from the spent medium was separated on a DEAE-cellulose column (40-mL bed volume) employing a linear gradient of 400 mL each of 0.01 and 0.25 M pyridine acetate, pH 5.2. At the end of the gradient, the glycopeptide material still bound to the column was eluted with 2 M pyridine acetate, pH 5.2. The volume of sample applied to the column was 6 mL, and fractions of 6 mL were collected. A column of 20-mL bed volume and a gradient of 200 mL each of 0.01 and 0.25 M pyridine acetate were employed for fractionating the cell-associated glycopeptides. The sample volume was 2 mL, and fractions of 2.5 mL were collected.

† From the Department of Biological Chemistry and The Specialized Cancer Research Center, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033. *Received June 4, 1979.* This work was supported by U.S. Public Health Service Contract NO1-CB-63984 and Grant CA-15483 from the National Cancer Institute.

[1] Abbreviations used: CPC, cetylpyridinium chloride; GAG, glycosaminoglycan; WGA, wheat germ agglutinin; $RCA_{120}$, *Ricinus communis* agglutinin of molecular weight 120000; $RCA_{60}$, *R. communis* agglutinin of molecular weight 60000; DEAE, diethylaminoethyl; NeuNAc, *N*-acetylneuraminic acid.

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Wheat germ agglutinin was prepared from crude wheat germ according to the procedure of Nagata et al. (1974), using ovalbumin–Sepharose after the second ammonium sulfate fractionation (Marchesi, 1972). A single protein band with a molecular weight of 29 000 was exhibited by this preparation on polyacrylamide gel electrophoresis. Conjugation of WGA to Sepharose 4B was done as described by Allan et al. (1972). For the study of glycopeptides, an analytical WGA–Sepharose column of 3-mL bed volume and a preparative WGA–Sepharose column of 25-mL bed volume were used. In conjunction with WGA–Sepharose, $RCA_{120}$–agarose and $RCA_{60}$–agarose obtained from Vector Laboratories (Burlingame, CA) were also employed for the characterization of glycopeptides.

*Paper Chromatography.* Descending paper chromatography was carried out on Whatman No. 1 paper with the following solvent systems: (a) 1-butanol–pyridine–water (6:4:3), (b) ethyl acetate–pyridine–water (12:5:4), (c) *tert*-amyl alcohol–2-propanol–water (8:2:3), (d) butyl acetate–acetic acid–water (3:2:1). Marker sugars were located by alkaline silver nitrate (Trevelyan et al., 1950) and sugar alcohols by periodate–benzidine (Gordon et al., 1956). Radioactivity was located by cutting paper chromatograms into 1-cm segments, shaking for 15 min in vials with 4 mL of water, adding 10 mL of Aquasol II (New England Nuclear Co., Boston, MA), and counting in a liquid scintillation spectrometer.

*Column Chromatography.* Biogel P6 columns (1.06 × 104 cm) equilibrated in 0.1 M pyridine acetate, pH 5.3, were used to study the products when the glycopeptides and the oligosaccharide alcohol were treated with glycosidases or 0.05 N $H_2SO_4$. In each experiment, fractions of 1.1 mL were collected; recoveries were generally more than 95%. Radioactivity was measured in the effluent fractions by mixing an 0.9-mL aliquot with 10 mL of scintillation cocktail containing xylene and Triton X-114 and counting in an Intertechnique Model SL36 spectrometer equipped with a disintegrations per minute calculating module. For preparative purposes, a Biogel P6 column (2.4 × 95 cm) equilibrated in 0.1 M pyridine acetate, pH 5.3, was used. In this case, 4.5-mL fractions were collected.

*Enzyme Digestions.* Enzymatically catalyzed hydrolysis of glycopeptides and the oligosaccharide alcohol was performed with highly purified glycosidases. The purity and activity of each enzyme were checked with appropriate *p*-(nitrophenyl)glycosyl derivatives, and each was established to be free of contaminating glycosidases. Digestion with β-galactosidase (*A. niger* or jack bean) and β-*N*-acetylglucosaminidase (jack bean) or β-*N*-acetylhexosaminidase (*D. pneumoniae*) was carried out for 20 h in microtubes with 0.5 unit of the enzyme in 50 mM phosphate–citrate, pH 5.1; the total incubation volume was 200 μL. Phosphate—citrate buffers of pH 7.0 and 6.6 were used, respectively, for digestion with β-galactosidase from *D. pneumoniae* and α-galactosidase from green coffee bean. Phosphate–citrate buffers of pH 7.0 and 6.6 were used, respectively, for digestion with β-galactosidase from *D. pneumoniae* and α-galactosidase from green coffee bean.

*Alkaline Borohydride Treatment of Glycopeptides.* Glycopeptides were incubated in glass-stoppered tubes for 20 h at 45 °C in the dark in 2 M $NaBH_4$–0.1 N NaOH. After incubation, excess borohydride was destroyed by careful addition of acetic acid to pH 4.5 (Maye & Carlson, 1970).

## Results

*Isolation and Fractionation of Glycopeptides.* The resolution of the spent medium glycopeptide material on the DEAE-cellulose column is illustrated in Figure 1. All three cell lines gave several distinct fractions (A–F) with the corresponding peaks between the cell lines occupying similar elution positions. The first peak was virtually devoid of sialic acid and was considered a neutral glycopeptide fraction; material eluting from the column during the gradient was presumed to be less anionic than that removed with 2 M pyridine acetate. The latter fraction is clearly more prominent in the cancer lines (Figure 1), and the MCF-7 line synthesizes more neutral glycoproteins as well. The cell-associated glycopeptide fractions did not give such distinct peaks, but the overall patterns were similar. The 2 M pyridine acetate fractions from spent medium were chromatographed on WGA–Sepharose.

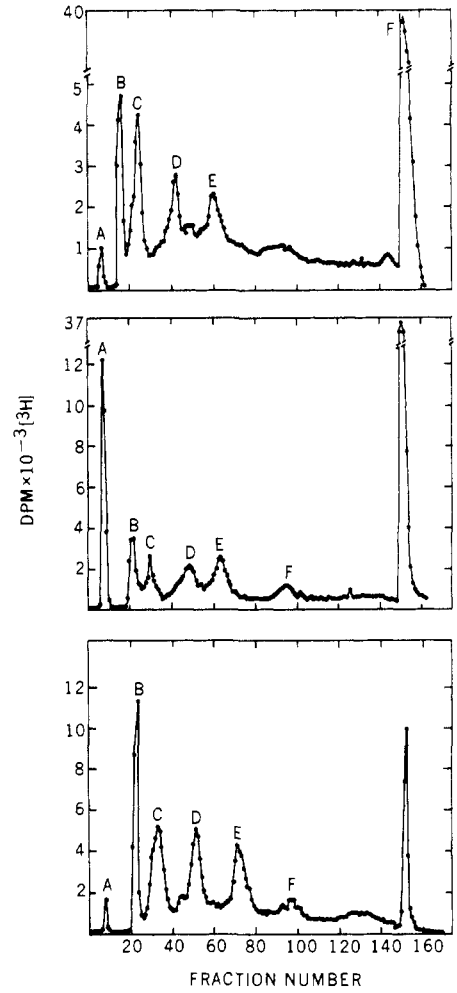


FIGURE 1: Elution profile on DEAE-cellulose of glycopeptides from spent medium obtained from cells grown in the presence of [$^3$H]-glucosamine. (Upper panel) Material from MCF-7 cells; (center panel) material from MDA-MB-231 cells; (lower panel) material from HBL-100 cells. The components eluting at about fraction 150 represent those displaced by 2 M pyridinium acetate.

The elution profiles (Figure 2) show that the MCF-7 fraction contained the highest proportion of WGA-bound material. The distribution of radioactivity from [$^3$H]glucosamine in cell-associated and spent-medium glycopeptide fractions is summarized in Table I. The results indicate that both cancer lines synthesize more anionic glycopeptides than do the HBL-100 cells and that within this fraction wheat germ agglutinin interacts with a significant proportion of the total.

*Characterization of WGA-Binding Glycopeptides.* The glycopeptide materials isolated from the three cell lines were found to be fairly homogeneous as judged by linear gradient elution with pyridine acetate from a DEAE-Sephadex column. When they were subjected to alkaline borohydride treatment to eliminate oligosaccharide chains linked O-glycosidically to

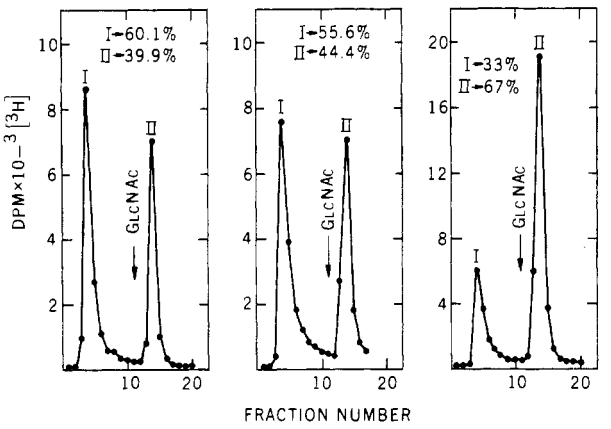


FIGURE 2: Fractionation of the anionic (2 M pyridinium acetate) glycopeptides on WGA–Sepharose. Elution with 0.1 M *N*-acetylglucosamine was initiated at the position marked with the arrow. (Left panel) HBL-100; (center panel) MDA-MB-231; (right panel) MCF-7.

Table I: [³H]Glucosamine Incorporation into Glycopeptides[a]

| glycopeptides (cell + medium) | radioact (dpm × $10^{-6}$/$10^7$ cells) HBL-100 | MDA-MB-231 | MCF-7 |
|---|---|---|---|
| neutral | 1.3 | 0.7 | 5.3 |
| less anionic | 31.9 | 44.4 | 26.9 |
| more anionic | 5.1 | 23.1 | 14.0 |
| WGA binding | 2.2 | 10.1 | 9.0 |

[a] Incorporation of [³H]glucosamine into glycopeptides. Cells were exposed to precursor for 48 h and products fractionated as described in the text. The neutral fraction is that not retained by DEAE-cellulose (peak A, Figure 1), the less anionic fraction is the composite of material eluted by the pyridine acetate gradient, and the more anionic fraction is that eluted by 2 M pyridine acetate.

serine or threonine residues, both cancer lines yielded a predominant oligosaccharide product. This appeared larger than the tetrasaccharide alcohol isolated from fetuin

NeuNAc–Gal–Gal(NeuNAc)NAcOH

and was marginally present if at all in the HBL-100 cells. A neutral, N-glycosidic glycopeptide fraction isolated from MCF-7 cells (DEAE-cellulose peak A), whose hexosamine was mostly glucosamine (>95%), served as a control in the alkaline elimination experiment. It did not yield any oligosaccharide alcohol, nor did its elution profile on Biogel P6 change.

The alkaline borohydride elimination product from the anionic glycopeptides was isolated by preparative column chromatography and purified by rechromatography on the same column (Figure 3). In the same manner, the major elimination product was also isolated from the MCF-7 WGA-binding glycopeptide which had been previously desialylated by incubation with neuraminidase and the analogous sialo- and asialosugar alcohols prepared from MDA-MB 231 culture. The four oligosaccharides were hydrolyzed in vacuo for 18 h at 100 °C with 4 N HCl.

The amino sugars and alcohols were separated by ion-exchange chromatography on the amino acid analyzer (Feldhoff et al., 1979). There were comparable amounts of radioactivity in glucosamine and galactosaminitol for the sialo- and asialooligosaccharides produced by both cell types; radioactivity in galactosamine was negligible. These data indicate that galactosamine is O-glycosidically linked to the peptide and that the oligosaccharide chain contains 1 mol of glucosamine per mol of galactosamine.

*Structure of the Major Oligosaccharide Chain.* Details are presented for material from MCF-7 cells but are substantially

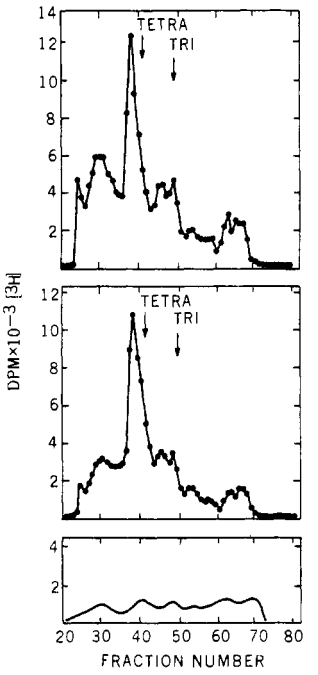


FIGURE 3: Elution profile on Biogel P6 of anionic, WGA-bound glycopeptides treated with alkaline borohydride. The elution positions of NeuNAc–Gal–Gal(NeuNAc)NAcOH (Tetra) and NeuNAc–Gal–GalNAcOH (Tri) standards are indicated by the arrows. The major peak (fractions 35–44) was combined for the MCF-7 (upper panel) and MDA-MB-231 (center panel) materials and concentrated. Rechromatography on the same column yielded a single peak with an unchanged elution position. Insufficient material was obtained from the HBL-100 cells (lower panel) to permit further analysis.

the same for the MDA-MB-231 cells. The major oligosaccharide from the sialo and asialo chains was isolated from MCF-7 cells labeled with [³H]glucosamine alone or with [³H]glucosamine and [¹⁴C]galactose. Paper chromatography of the sialooligosaccharide showed a single component with a mobility slower than that of the fetuin tetrasaccharide alcohol. The ³H and ¹⁴C labels were coincident, indicating the presence of galactose in the oligosaccharide chain; acid hydrolysis followed by paper chromatography confirmed that the ¹⁴C label was exclusively in galactose.

The asialooligosaccharide preparations labeled as above moved as single components and to an identical position when examined by paper chromatography in two different solvent systems (Figure 4). Based on this result, it was assumed that the two asialo compounds (³H or ³H/¹⁴C labeled) were identical and suitable for subsequent structural analysis.

Treatment of the ³H-labeled asialooligosaccharide with β-*N*-acetylhexosaminidase (from *D. pneumoniae* or jack bean) was followed by chromatography on Biogel P6. The mobility was unchanged from that of the starting material nor was any degradation product detected, suggesting the absence of terminal *N*-acetylglucosamine. On the other hand, when the oligosaccharide preparation (³H and ¹⁴C labeled) was treated with β-galactosidase (*D. pneumoniae*) and then chromatographed on the Biogel P6 column, 50% of the ¹⁴C label was liberated and the remaining ¹⁴C label was associated with all of the ³H label (Figure 5). The elution position of the ³H- and ¹⁴C-containing component was more included than that of the starting material, suggesting the hydrolysis of one of two galactose moieties by the β-galactosidase. The slower moving component emerging from the Biogel P6 column was identified as galactose by paper chromatography in solvent systems a, b, and c. The remaining material, which had a higher mobility than the parent compound, migrated as a single

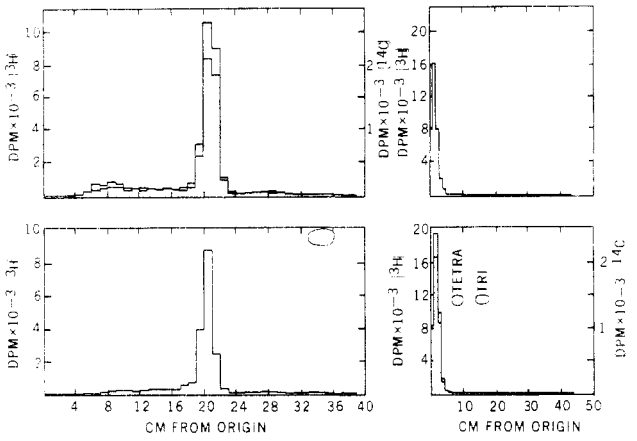


FIGURE 4: Paper chromatography of oligosaccharides isolated from MCF-7 spent medium glycopeptides. Right panels are the sialo-oligosaccharide alcohol (Figure 3) from cells grown in the presence of [$^3$H]glucosamine (upper) or [$^3$H]glucosamine and [$^{14}$C]galactose; Tetra and Tri refer to marker standards NeuNAc–Gal–Gal(NeuNAc)NAcOH and NeuNAc–Gal–GalNAcOH, respectively. Left panels are the corresponding asialo products from [$^3$H]glucosamine (upper) or [$^{14}$C]galactose and [$^3$H]glucosamine (lower) grown cells; the shaded marker in the lower chromatogram is malotriose. Chromatography was carried out by using solvent d for 24 h (sialo-oligosaccharide) and solvent b for 48 h (asialooligosaccharide).

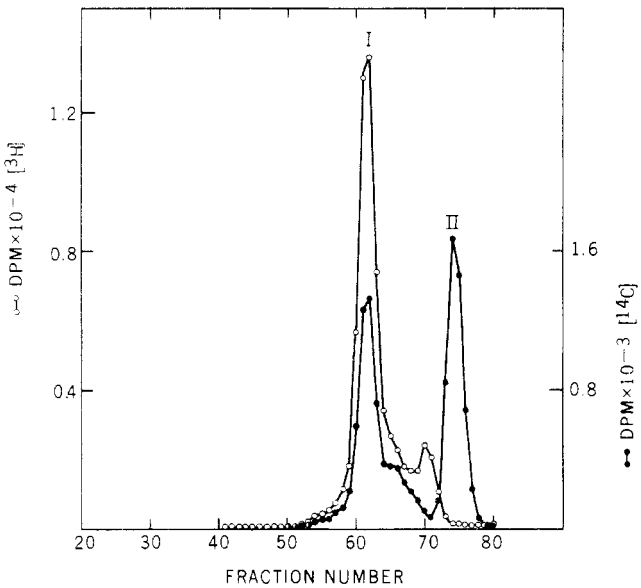


FIGURE 5: Biogel P6 chromatography of products obtained by $\beta$-galactosidase digestion of the $^3$H/$^{14}$C-labeled asialooligosaccharide alcohol (Figure 4). Peaks I and II correspond to a trisaccharide and galactose, respectively. See text for details.

substance on paper chromatography. These results suggest that the sugar sequence in the asialooligosaccharide chain may be Gal$\overset{\beta}{\rightarrow}$GlcNAc$\rightarrow$Gal$\rightarrow$GalNAcOH.

Reaction of the doubly labeled asialooligosaccharide with $\beta$-galactosidase followed by $\beta$-*N*-acetylhexosaminidase and by Biogel P6 chromatography gave four products. Two contained both $^3$H and $^{14}$C labels, a third only $^3$H, and a fourth only $^{14}$C. These peaks apparently represent a trisaccharide (GlcNAc–Gal–GalNAcOH), a disaccharide (Gal–GalNAcOH), *N*-acetylglucosamine, and galactose, respectively.

The asialoagalacto product (peak I of Figure 5) was digested with $\beta$-*N*-acetylglucosaminidase, and the products were separated by chromatography on Biogel P6. Two fractions were obtained; one had both $^3$H and $^{14}$C labels and the other had ~50% of the $^3$H label (Figure 6). The $^3$H-labeled material was identified as *N*-acetylglucosamine by paper chromatography. The other fraction contained mainly a disaccharide and some undigested asialoagalactooligosaccharide.

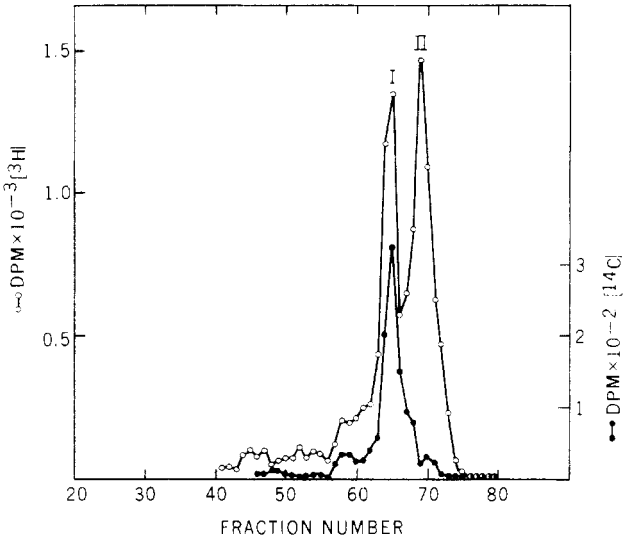


FIGURE 6: Biogel P6 chromatography of products obtained by $\beta$-*N*-acetylglucosaminidase digestion of the trisaccharide alcohol (peak I of Figure 5). Peak II was identified as *N*-acetylglucosamine.

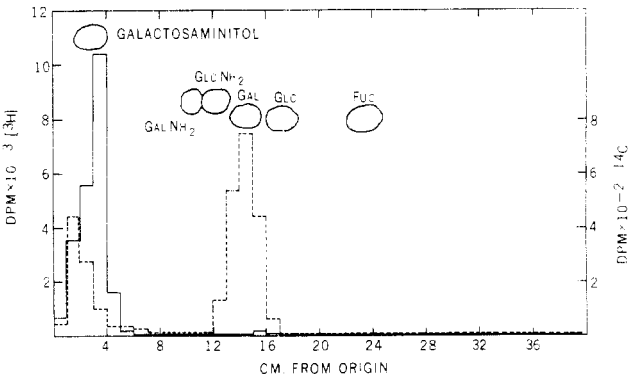


FIGURE 7: Paper chromatography of products obtained by acid hydrolysis of the oligosaccharide alcohol remaining after successive treatment with sialidase, $\beta$-galactosidase, and $\beta$-*N*-acetylglucosaminidase (peak I of Figure 6). The solid line represents $^3$H radioactivity and the broken line $^{14}$C radioactivity. Marker saccharides in addition to galactosaminitol are galactosamine ($GalNH_2$), glucosamine ($GlcNH_2$), galactose (Gal), glucose (Glc), and fucose (Fuc). Chromatography was for 24 h using solvent a.

The disaccharide product was resistant to both $\alpha$- and $\beta$-galactosidase. However, acid hydrolysis followed by paper chromatography yielded a $^{14}$C-labeled monosaccharide identified as galactose and a $^3$H-labeled monosaccharide identified as galactosaminitol (Figure 7).

The same results were obtained when the asialooligosaccharide was digested with $\beta$-galactosidase (*A. niger*) + $\beta$-*N*-acetylglucosaminidase (jack bean), $\beta$-galactosidase (jack bean) + $\beta$-*N*-acetylglucosaminidase (jack bean), or $\beta$-galactosidase (jack bean) + $\alpha$-galactosidase (coffee bean) + $\beta$-*N*-acetylglucosaminidase (jack bean).

The possibility remained that the resistance of the internal galactosyl moiety to enzymatic hydrolysis could also be explained by its substitution by an additional sugar not revealed by the metabolic labeling approach thus far adopted. Since the only sugar fulfilling these requirements was fucose, both cancer lines were exposed to [$^3$H]fucose in experiments analogous to those performed with [$^3$H]glucosamine. Although considerable label was incorporated into the glycopeptide fractions, less than 1% was present in the most anionic region of the DEAE-cellulose column. Accordingly, the failure of

Table II: Degradation of $^3H/^{14}C$-Labeled Oligosaccharide Alcohol

| | oligo alcohol | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| sialidase | NeuNAc + II | no reaction | | |
| β-galactosidase | no reaction | Gal (50%) + III | no reaction | no reaction[a] |
| β-*N*-acetylglucosaminidase | no reaction | no reaction | *N*-AcGlcNH$_2$ + IV | no reaction |
| $H^+$ | NeuNAc, Gal, GlcNH$_2$, GalNH$_2$OH | Gal, GlcNH$_2$, GalNH$_2$OH | Gal, GlcNH$_2$, GalNH$_2$OH | Gal, GalNH$_2$OH |

[a] α-Galactosidase digestion had no effect on IV.

either α- or β-galactosidase to release galactose from the saccharide alcohol IV cannot be ascribed to substitution by a fucosyl residue. The enzymatic and chemical data are summarized in Table II.

*Glycopeptide Characteristics.* The double-labeled wheat germ bound glycopeptide obtained from MCF-7 cells labeled with [$^{14}$C]glucosamine and [$^3$H]galactose contained 27.3% sialic acid, as estimated by release of radioactivity (identified as NeuNAc) after hydrolysis for 1 h at 80 °C with 0.05 N $H_2SO_4$; no other radioactive peak in the position of monosaccharide was detected. The asialoglycopeptide was resistant to β-*N*-acetylglucosaminidase (jack bean). On the other hand, incubation of the asialoglycopeptide with β-galactosidase released a portion of the $^3$H label as free galactose with the remainder as peptide–oligosaccharide which retained all of the $^{14}$C label. The anionic glycopeptide fraction which did not bind to WGA–Sepharose contained 25.2% of its $^{14}$C label in sialic acid. The asialo fraction was resistant to β-*N*-acetylglucosaminidase whereas digestion with β-galactosidase released 23.5% of the $^3$H label as galactose.

These data show that the sialic acid content of both the WGA-bound and -unbound fractions is similar and that the asialoglycopeptide fractions have terminal β-galactosyl residues. The combined action of β-galactosidase and β-*N*-acetylglucosaminidase released more *N*-acetylglucosamine (36.3% of $^{14}$C label as compared to 22.0%) from the WGA-binding glycopeptides than from the nonbound, indicating that the sugar chains Gal$\overset{\beta}{\rightarrow}$GlcNAc$\overset{\beta}{\rightarrow}$Gal→GalNAc are more abundant in the former. The failure to release all of the $^3$H label even after a second treatment with these enzymes raises the possibility that the glycosidic linkage of the interior galactose is different from the exterior one.

The cell-associated WGA-bound glycopeptide fraction from the MDA-MB-231 line contained 32.1% sialic acid (NeuNAc) as estimated by Biogel P6 chromatography after hydrolysis in 0.05 N $H_2SO_4$. The asialoglycopeptide did not bind to WGA–Sepharose but completely bound to an $RCA_{60}$– or $RCA_{120}$–agarose column, indicating that a β-galactosyl residue is the terminal sugar in these oligosaccharide chains. Since digestion of this material with β-galactosidase and β-*N*-acetylglucosaminidase released *N*-acetylglucosamine as well as galactose, it is suggested that most of the oligosaccharide chains present have as a partial or complete sequence Gal$\overset{\beta}{\rightarrow}$GlcNAc$\overset{\beta}{\rightarrow}$Gal→GalNAc. Essentially identical characteristics were exhibited by the corresponding MCF-7 fraction.

The corresponding WGA-nonbound glycopeptide from the MDA cells had 36.2% sialic acid but, based on exclusion chromatography, had a lower molecular weight. About 80% of this material bound to RCA–agarose.

A comparison of WGA-binding and -nonbinding glycopeptides illustrates the following requirements for WGA binding: (a) the molecular size must be relatively large (6000 daltons or greater); (b) several clustered oligosaccharide chains having a terminal sequence Gal→GlcNAc→ must be present; (c) most of the exterior galactosyl residues must contain a sialyl

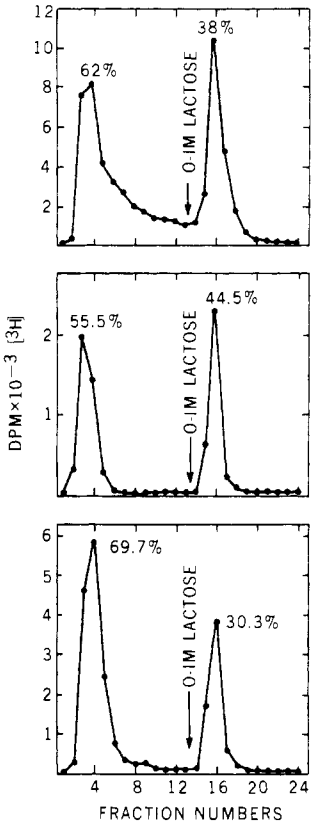


FIGURE 8: Lectin affinity chromatography of anionic glycopeptides not bound to WGA–Sepharose. (Upper panel) MDA-MB-231 spent-medium fraction on $RCA_{120}$–agarose; (middle panel) MDA-MB-231 spent-medium fraction on $RCA_{60}$–agarose; (lower panel) MCF-7 spent-medium fraction on $RCA_{120}$–agarose.

substituent. Although both types of glycopeptides contain approximately the same amount of sialic acid (32–36%), in view of the fact that the asialo material does not bind to WGA–Sepharose, the number and arrangement of the sialic acid residues clearly play a critical role in binding to WGA (Bhavanandan & Katlic, 1979).

The WGA-bound glycopeptides isolated from the spent medium of the MDA-MB-231 cells had 28.7% sialic acid; the asialo product did not bind to WGA–Sepharose but bound completely to $RCA_{60}$– or $RCA_{120}$–agarose, indicating that a β-galactosyl residue is now at the nonreducing terminus. Digestion with β-galactosidase and β-*N*-acetylglucosaminidase released both galactose and *N*-acetylglucosamine, showing an overall structure similar to that of the corresponding cell-associated glycopeptide.

The WGA-nonbound glycopeptide contained 22.1% sialic acid, and only 38% of the asialo material from the MDA cells bound to $RCA_{120}$–agarose (Figure 8). These data suggest that several saccharide structures are present and/or that the organization of these sequences on the polypeptide chain differs from that in the WGA-bound fraction. The requirements for WGA binding are evidently quite similar to those noted for the cell-associated glycopeptide.

Table III: Amino Sugar Composition of WGA-Bound and Nonbound Glycopeptides[a]

| glycopeptide fraction | glucosamine | galactosamine | sialic acid |
|---|---|---|---|
| WGA bound | | | |
| MDA (media) | 36.0 | 32.0 | 32.0 |
| MCF (media) | 36.0 | 33.0 | 30.6 |
| MDA (cell) | 37.4 | 30.6 | 32.0 |
| WGA nonbound | | | |
| MDA (media) | 63.8 | 21.2 | 15.0 |
| MCF (media) | 47.5 | 24.5 | 28.0 |
| HBL (media) | 46.8 | 38.2 | 15.0 |
| MDA (cell) | 38.7 | 29.3 | 32.0 |

[a] Proportion of $^3H$ label from [$^3H$]glucosamine incorporated into glucosamine, galactosamine, and sialic acid.

Analyses of the MCF-7 cell-associated and spent-medium anionic glycopeptides gave results analogous to those obtained for the MDA-MB-231 materials. In general, the WGA-bound fractions were of higher molecular weight than those that did not bind to the lectin, had more chains of terminal sequence Gal$\overset{\beta}{\rightarrow}$GlcNAc, and contained a slightly greater proportion of sialyl residues, which is a component essential for binding to occur. Analytical data are summarized in Table III.

## Discussion

A major component of the total glycopeptide production of both the MCF-7 and MDA-MB-231 cell lines is the anionic material eluted with 2 M pyridine acetate from the DEAE-cellulose column. Although this fraction is heterogeneous, virtually all of the saccharide units are O-glycosidically linked to the peptide and the predominant structure appears to be a pentasaccharide. The composite enzymatic, analytical, and chromatographic data obtained on the oligosaccharide alcohol are consistent with the linear sequence NeuNAc$\overset{\alpha}{\rightarrow}$Gal$\overset{\beta}{\rightarrow}$GlcNAc$\overset{\beta}{\rightarrow}$Gal$\rightarrow$GalNAcOH. The linkage configurations are established with the exception of that between the internal galactose and the *N*-acetylgalactosaminitol. Based on analogy with other glycoproteins of this type and the resistance of this galactosidic bond to $\alpha$-galactosidase digestion, we assume the linkage to be $\beta(1\rightarrow3)$. This presumption is supported by the susceptibility of the partially digested glycopeptide to the *Diplococcus endo-α-N*-acetylgalactosaminidase whose specificity has been previously described (Umemoto et al., 1977). The presence of this saccharide unit in glycoproteins derived from HBL-100 cells could not be clearly established. On the basis of detection sensitivity, this structure occurs one or more orders of magnitude less frequently in the HBL-100 cells than it does in either of the cancer lines. It is of interest that the proportion of both highly anionic saccharides and the pentasaccharide unit is nearly identical for the MCF-7 and MDA-MB-231 cells. Furthermore, the sequence and structure of this unit from the two cell lines appear identical.

A second structural parameter is the distribution, both frequency and spacing, of the oligosaccharide units on the parent glycoprotein. Molecular weight estimates of the products obtained from either cancer cell line after exhaustive Pronase digestion suggest that four to six pentasaccharide moieties are closely spaced. This conclusion is reinforced by the lectin affinity characteristics of the glycopeptides. Prior studies from our laboratory have established that clustered sialyl residues represent a major ligand site for wheat germ agglutinin, although structures with a single nonreducing sialic acid terminus interact poorly with the lectin (Bhavanandan & Katlic, 1979). Removal of the sialyl groups results in complete loss of affinity for WGA–Sepharose. The resulting asialoglycopeptide binds to both of the galactose ligands from *Ricinus communis* ($RCA_{120}$ and $RCA_{60}$).

The high density of saccharide substitution is not unknown for mucin-type glycoproteins but has generally been associated with secretory products of epithelial cells. The occurrence of such structural features in products of mouse and human melanoma cells (Bhavanandan et al., 1977; V. P. Bhavanandan and E. A. Davidson, unpublished experiments) as well as the mammary cancer lines suggests a commonality that may be associated with the malignant phenotype.

The outer sequence NeuNAc$\rightarrow$Gal$\rightarrow$GlcNAc is a commonly occurring one in glycoproteins. However, it is most often found on asparagine-linked, mannose-containing complex oligosaccharides. The O-glycosidically linked structure biosynthesized by the two mammary carcinoma lines is unusual, although a comparable unit may occur as a minor constituent of epiglycanin, the characteristic glycoprotein of the TA3 murine mammary carcinoma (Codington et al., 1975). As with the frequency of the oligosaccharide substitution sites, the architecture of the sugar chain may reflect the growth potential of the cell.

The presence of similar glycopeptides in the cell-associated fraction raises the question of membrane involvement. Whether the material present in the culture medium arises by routine membrane turnover or as a result of surface proteolysis cannot be determined at this time.

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