Isolation and Partial Characterization of Sialoglycopeptides Produced by a Murine Melanoma†

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ABSTRACT: The glycopeptides produced by B16 mouse melanoma cells grown in the presence of [^{3}H]glucosamine and [^{35}S]sulfate were isolated from the cells and from spent media. Treatment of the media and cells with pronase gave a mixture of isotopically labeled glycopeptides and glycosaminoglycans. One class of sialoglycopeptides was fractionated from this mixture by precipitation with cetylpyridinium chloride and purified by porous glass bead chromatography and ion exchange chromatography. It possessed greater molecular weight and charge than those glycopeptides which are not precipitated with cetylpyridinium chloride. This sialoglycopeptide has tetra- and trisaccharide moieties which contain sialic acid, *N*-acetylgalactosamine, and galactose attached *O*-glycosidically, apparently in clusters, to the peptide core. A notable feature was that this sialoglycopeptide, which is devoid of *N*-acetylglucosamine, was adsorbed on a wheat germ agglutinin–Sepharose column and also had a strong inhibitory activity on the wheat germ agglutinability of the B16 melanoma cells. The interaction between this sialoglycopeptide and wheat germ agglutinin was found to be due to the presence of several sialyl oligosaccharides attached to the same peptide backbone in the molecule. This mucin-type sialoglycopeptide, which has features common with human erythrocyte membrane glycoprotein, appears to be produced by several malignant cell lines, including the one investigated in the present studies.

Circumstantial evidence has implicated surface carbohydrates in cellular functions such as cell–cell recognition, adhesion, maintenance of membrane integrity, regulation of cell growth, and receptor sites. Recently, there have been a number of reports that a "cell-surface" glycoprotein of 200 000–250 000 daltons is present on several normal cells but is absent or diminished in the corresponding transformed cells (Hynes, 1973; Gahmberg et al., 1974; Yamada and Weston, 1974; Graham et al., 1975). Such changes of cell-surface carbohydrate have been attributed to increased proteolytic activity manifested at the surfaces of transformed cells (Schnebli and Burger, 1972; Ossowski et al., 1973), but definitive proof for this hypothesis is lacking. However, if this were the case, one would expect to detect glycopeptides arising from the high-molecular-weight and other cell-surface glycoproteins in the spent media in which transformed cells are grown. In the animal, a large amount of these glycopeptides may be shed by the neoplastic cells into the circulation. Such shedding may be a possible mechanism by which these cells escape the immunosurveillance of the host or it may be a factor in the poor adherence of these cells and their consequent metastasizing potential (Sjögren et al., 1971; Kim et al., 1975).

Antigens associated with B16 melanoma which appear to be secreted or shed by the cells have been isolated from spent media in which cells were grown in the presence of [^{3}H]leucine (Bystryn et al., 1974; Bystryn, 1976). Biochemical characterization of these antigens has not been done, but they appear to be glycoproteins.

In earlier reports we have discussed the nature of glycosaminoglycans produced by several cell lines (Satoh et al., 1973, 1974; Banks et al., 1976; Bhavanandan and Davidson, 1975, 1977). This study describes the isolation and structural characterization of one class of sialoglycopeptides produced by mouse melanoma cells. A preliminary report has been published (Bhavanandan and Davidson, 1976).

Experimental Procedure

Materials. The B16 melanotic melanoma cell lines studied were the second and third clones (B16C2 and B16C3) sequentially isolated by Kreider et al. (1973). An amelanotic clone isolated from stock B16 tumor maintained by the Jackson laboratory, Bar Harbor, Maine, was also used. Both the melanotic and amelanotic cell lines produce similar glycoconjugates (Satoh et al., 1974). Components for culture media, with the exception of antibiotic solution and fetal calf serum, were obtained from Grand Island Biological Co., Grand Island, N.Y. Pronase and neuraminidases (*Vibrio cholerae* and influenza virus) were obtained from Calbiochem; controlled pore glass beads (CPG[1] 10-240) were from Electronucleonics, Fairfield, N.J.; Sephadex G-50 was from Pharmacia; Sepharose 4B, DEAE[1]-Sephadex, β-galactosidase (*E. coli*), ovalbumin, and fetuin were from Sigma; cyanogen bromide was purchased from Eastman Kodak; DEAE-cellulose (DE-52) was from Reeve Angel; mixed glycosidases, α-*N*-acetylgalactosaminidase, and β-hexosaminidase (all from *Charonia lampis*) were purchased from Miles Laboratories; Tos-PheCH$_2$Cl-trypsin was obtained from Worthington Biochemical Corp. *endo*-α-*N*-Acetylgalactosaminidase and β-hexosaminidase from *Diplococcus pneumoniae* were isolated as described (Bhavanandan et al., 1976; Umemoto et al., 1977). Jack bean β-galactosidase was a gift from Dr. Y. T. Li.

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[1] Abbreviations used are: Con A, concanavalin A; RCAI, *Ricinus communis* agglutinin (mol wt 120 000); RCAII, *Ricinus communis* agglutinin (mol wt 60 000); CPC, cetylpyridinium chloride; Tos-PheCH$_2$Cl-trypsin, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin; NANA, *N*-acetylneuraminic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; DEAE, diethylaminoethyl; CPG, controlled pore glass beads; WGA, wheat germ agglutinin.

Wheat germ agglutinin (WGA) was isolated from crude wheat germ according to Nagata et al. (1974), except that after the second ammonium sulfate fractionation an affinity column of ovalbumin-Sepharose was used for purification (Marchesi, 1972). The preparation gave a single band on polyacrylamide gel electrophoresis and had an apparent molecular weight of 29 000. Concanavalin A (Con A) was prepared from Jack bean meal (Sigma) as described by Agrawal and Goldstein (1972). The agglutinins RCAI (120 000 mol wt) and RCAII (60 000 mol wt) from *Ricinus communis* were isolated as described by Nicolson and Blaustein (1972), except that the seeds were first crushed, defatted with cold acetone (−20 °C) and air-dried before extraction with phosphate buffer. Soybean agglutinin was isolated from defatted soybean meal according to the procedure of Allen and Neuberger (1975); it should be noted that several commercial batches of soybeans and soybean meal that were tested did not have any activity. Conjugation of the lectins to Sepharose 4B was done essentially as described by Allan et al. (1972); the remaining active groups were blocked by treatment with 0.1 M ethanolamine hydrochloride in 1 M NaCl-0.1 M $NaHCO_3$, pH 8.0, by stirring for 8 h at 4 °C. The product was packed in a column and washed successively with 0.1 M $NaHCO_3$; 0.1 M $NaHCO_3$-1 M NaCl, pH 8.0; 0.1 M sodium acetate-1 M NaCl, pH 4.0; distilled water; 0.1% bovine serum albumin in 0.05 M Tris-HCl, pH 8.0; and finally with 0.05 M Tris-HCl with 10 mM Ca^{2+}, 1 mM Mg^{2+}, 1 mM Mn^{2+}, pH 7.0, for the Con A column and 0.05 M Tris-HCl, pH 8.0, for all other lectin columns.

Column Chromatography. Controlled pore glass 10-240 beads of 80/120 mesh were treated with poly(ethylene glycol) and packed with constant vibration according to the instruction of the manufacturers. The columns were equilibrated with 0.5 M $CaCl_2$ or 0.5 M KCl and eluted with the same solution using a pump to maintain a constant flow rate of 30 ml/h. Sephadex G-50, Bio-Gel P2 (200–400 mesh), and P4 and P6 columns were equilibrated and eluted with 0.1 M pyridine acetate, pH 5.0. DEAE-cellulose (DE-52 microgranular) was degassed by suspending in 1.0 N acetic acid (pH 3.0), neutralized with pyridine to pH 5.2, and filtered through a sintered glass funnel. The ion exchanger was then equilibrated by washing extensively with 1.0 M pyridine acetate and packed in a column (0.9 × 70 cm). The sample was applied in 0.01 M pyridine acetate and the column eluted with a linear gradient of 0.01 M pyridine acetate, pH 5.1, in the mixing chamber and an equal volume of 1.0 M pyridine acetate, pH 5.1, in the reservoir. Cellulose acetate electrophoresis was carried out in a Beckman R-101 microzone electrophoresis cell using 0.2 M calcium acetate, pH 7.0, at 5 mA, for 3 h or in 0.1 M pyridine–formic acid buffer, pH 3.0, at 10 mA for 20 min. Alcian blue (0.1% in 0.5% acetic acid), periodate-Schiff reagent, and Ponceau-S were used for staining.

Paper chromatography was carried out on Whatman no. 1 paper by the descending technique. The following solvent systems were employed: (A) 1-butyl acetate–acetic acid–water (3:2:1), (B) 1-butanol–pyridine–water (6:4:3), (C) pyridine–ethyl acetate–water–acetic acid (5:5:3:1) with pyridine–ethyl acetate–water (11:40:6) in the bottom of the chromatography tank, (D) 1-butanol–acetic acid–water (4:1:5, upper phase). Sialic acids were detected by the thiobarbituric acid spray (Warren, 1960). Neutral sugars, hexosamines, and oligosaccharides were located by the silver nitrate staining procedure (Trevelyan et al., 1950) and sugar alcohols by the periodate–benzidene technique (Gordon et al., 1956).

Hexosamine determinations on isotopically labeled components were carried out on acid hydrolysates on the amino acid analyzer by the stream-splitting technique described earlier (Bhavanandan and Davidson, 1976). Total sialic acid in isotopically labeled glycoproteins was determined either by acid hydrolysis (0.1 M H_2SO_4, 80 °C, 1 h) or neuraminidase treatment followed by separation of sialic acid from asialoglycoprotein on a Bio-Gel P2 column. Total sialic acid was determined by the periodate–resorcinol method (Jourdian et al., 1971). Marker polysaccharides run on gel columns were assayed by the orcinol reaction (Davidson, 1966). Liquid scintillation counting was performed on an Intertechnique Model SL36 spectrometer. Usually, 1-mL aqueous samples were mixed with 10 mL of the counting liquid containing xylene and Triton X-114 (Anderson and McClure, 1973). Efficiencies for ^3H and ^{35}S were about 16 and 47%, respectively, with a crossover of about 13–14% ^{35}S into the ^3H channel. The 4-mL citrate buffer fractions from the amino acid analyzer were mixed with 15 mL of 3a40 counting fluid (from Research Products International Corp.) and counted. The efficiency for tritium in this system was 15%, using the same settings for minimal ^{35}S crossover as above. Radioactivity on cellulose acetate or paper strips was estimated by extracting cut pieces with 1 mL of water in counting vials. After shaking for at least 6 h on a reciprocating shaker, counting liquid was added and the solution mixed and counted.

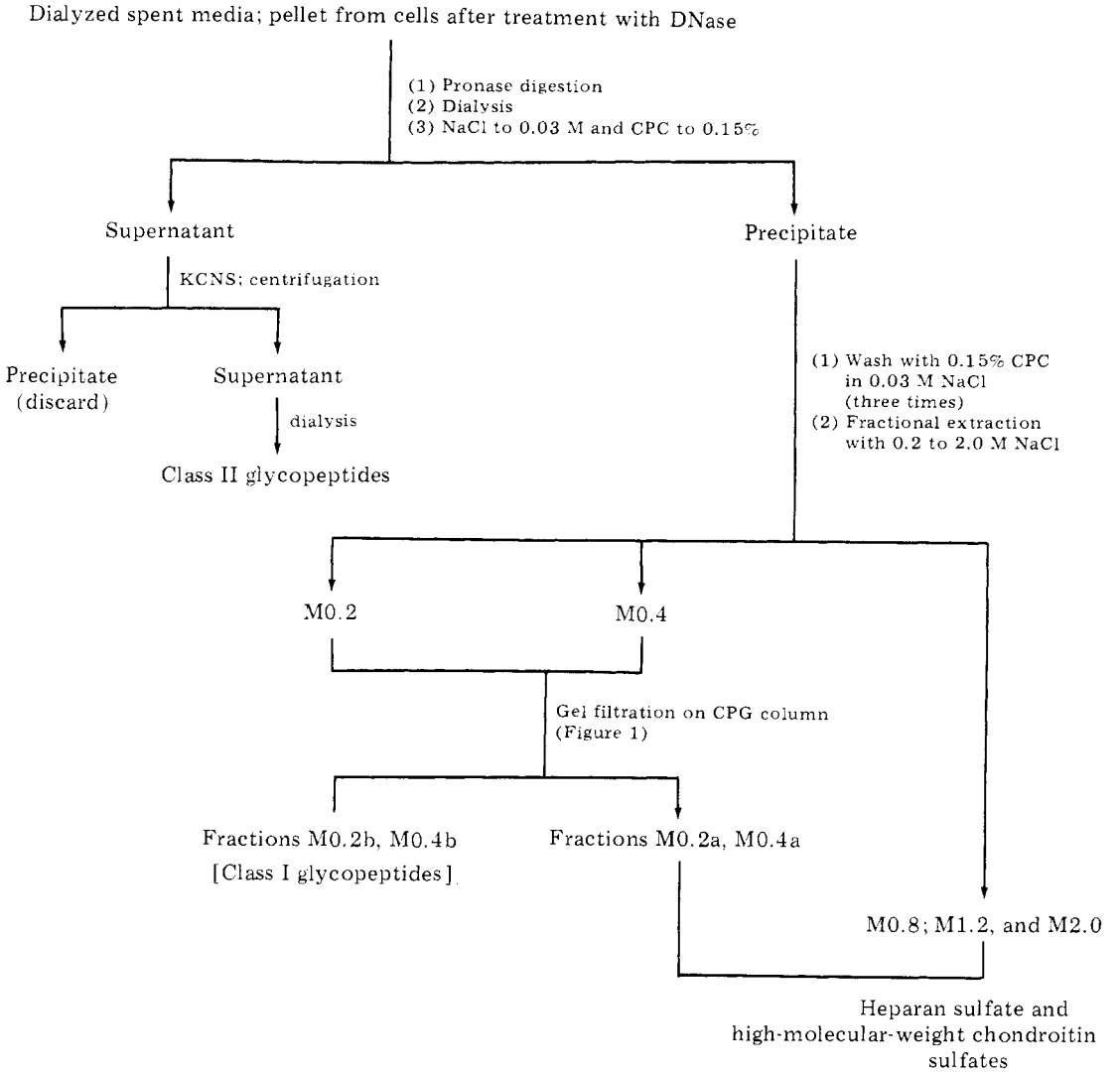
Digestion with Glycosidases. Vibrio cholerae neuraminidase digestion was done in 0.1 M sodium acetate buffer, pH 5.6, containing 0.001 M $CaCl_2$ using 0.1–1.0 unit of enzyme in a total digest volume of 200 μL; incubation was performed at 37 °C for the specified time. Influenza virus neuraminidase digestion was carried out in 0.05 M Tris–maleate buffer, pH 6.5, containing 0.001 M $CaCl_2$ using 0.1–0.2 unit of the enzyme in a volume of 200 μL incubated as above. The released product was determined by the modified thiobarbituric acid method (Yeh et al., 1971) in experiments using unlabeled oligosaccharide substrates. In experiments with labeled sialoglycopeptide, the released sugar was estimated either by separation on Bio-Gel P2 columns or by loss of radioactivity on dialysis.

Incubation with β-galactosidase from *Escherichia coli* or Jack Bean was done in citrate–phosphate buffer, pH 6.5, using 5 units of enzyme or citrate buffer, pH 4.0, using 0.2 unit enzyme, respectively, 37 °C for 48 h in a total volume of 150 μL. The *E. coli* enzyme had no action on *p*-nitrophenyl α-D-galactopyranoside, *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide, or *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide. Digestion with β-*N*-acetylhexosaminidase from *Charonia lampus* was done as for *E. coli* β-galactosidase but using 0.1 unit of enzyme; incubation with the β-hexosaminidase from *D. pneumoniae* was performed for 6 h at pH 5.3 with 100 milliunits of enzyme. The enzyme from *Charonia lampus* had ten times more activity toward *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as compared to *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide.

Digestion with α-*N*-acetylgalactosaminidase from *Charonia lampus* was done in 0.05 M citrate buffer, pH 4.0, using 5–10 milliunits of enzyme in a total volume of 100 μL at 37 °C for 24 h. Treatment with *endo*-α-*N*-acetylgalactosaminidase from *D. pneumoniae* (Bhavanandan et al., 1976) was done in Tris–maleate buffer, pH 7.0, or citrate phosphate buffer, pH 6.5, at 37 °C for 6–24 h in a total volume of 50 μL.

Alkaline Borohydride Treatment of Glycopeptides. Alkaline borohydride treatment of the glycopeptides was done with 1.0 M $NaBH_4$ or 0.3 M $NaBH_4$ in 0.1 N NaOH for 3–4 days at 37 °C under nitrogen in sealed tubes in the dark. The reaction mixture was cooled in an ice bath and the excess borohydride destroyed by careful addition of 1 N acetic acid. Isolation of tetra- and trisaccharides from fetuin by treatment with alkaline borohydride was as described by Spiro and

SCHEME I: Isolation of Glycopeptides Produced by Murine Melanoma Cells



Bhoyroo (1974).

Cell Cultures. B16 mouse melanoma cell lines (B16C2 and B16C3) and an amelanotic clone were routinely grown in 16-oz prescription bottles in minimum essential medium with Earle's salt solution supplemented with nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), twice the recommended concentration of vitamins (Eagle, 1959), 10% heat-inactivated fetal calf serum, and 10 units per mL of penicillin G and 10 μg per mL of streptomycin sulfate. The particular clone has been screened for mycoplasma contamination and its tumorigenicity established by ability to develop tumors in appropriate host mice. Cells were subcultured at confluency by suspension with 0.02% EGTA ([ethylenebis(oxyethylenenitrilo)]tetraacetic acid) in calcium magnesium free phosphate buffered saline. The spent media at each medium change and at subculturing were decanted, centrifuged to remove floating cells, and stored in the freezer until used. Labeled complex saccharides were prepared from cells and spent medium after culturing the cells for 48 h prior to harvest in medium containing 10 μCi of [^{3}H]glucosamine per mL (New England Nuclear, 755 mCi/mmol). For the identification of the neutral sugars, cells were grown in the presence of [^{3}H]glucose (New England Nuclear, 34 mCi/mmol) at 40 μCi per mL in F12 media (Ham, 1965) containing 1% bovine serum albumin (Banks et al., 1977).

Isolation of Labeled Glycopeptides from Spent Media and Cells (refer to Scheme I). Pooled frozen spent medium, about 5 L, was thawed and dialyzed at 4 °C against 0.9% NaCl for 2 days, followed by water for 5 days in the presence of toluene and chloroform. The dialyzed material was concentrated by ultrafiltration and lyophilization to about 600 mL and combined with 100 mL of dialyzed labeled media. Calcium acetate was added to a concentration of 0.01 M Ca^{2+} and the pH adjusted to 7.8 with 10 N NaOH. Pronase (100 mg) was stirred in, 2 mL of toluene added, and the solution incubated in a stoppered flask at 40 °C for 72 h with further additions of 100 mg of enzyme and toluene after 24 and 48 h. The pH was maintained at 7.8–8.0 during the incubation by addition of NaOH. The digest was centrifuged, the residue discarded, and the supernatant dialyzed against saline followed by deionized water for 4 days. The contents of the dialysis bag were adjusted to pH 12.5 and after 18 h at room temperature (20–25 °C) were dialyzed for 5 days against several changes of deionized water. A second batch of spent media (3 L) as well as the media from the [^{3}H]glucose experiment were worked up in an identical manner except that the treatment at pH 12.5 was omitted.

For isolation of cell-associated complex saccharides, the cells were first treated with DNase, and the insoluble pellet obtained digested with pronase to yield a mixture of soluble complex saccharides. The pronase digests were dialyzed and subjected to CPC precipitation.

The pronase- and alkali-treated material was adjusted to

TABLE I: Results of CPC Precipitation and Salt Elution of Pronase-Digested Spent Media.

Fraction	Expt I dpm × 10^{-6}	Expt I ^3H %	Expt I Wt (mg)	Expt II dpm × 10^{-6}	Expt II ^3H %	Expt II Wt (mg)
CPC supernatant	46.85	89.7		32.80	89.0	
0.2 M NaCl	2.28	4.4	7.4	0.64[a]	1.7	1.9[a]
0.4 M NaCl	0.40[a]	0.8	7.8[a]	2.40	6.5	7.1
0.8–2.0 M NaCl	2.68	5.1	16.3	1.01	2.7	8.3

[a] Some of these fractions were lost accidentally during dialysis.

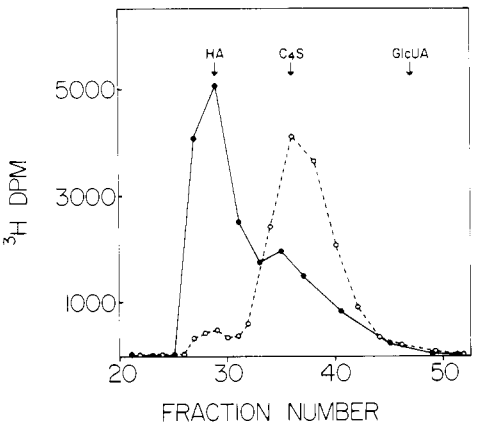


FIGURE 1: Fractionation of 0.2 (broken line) and 0.4 M (solid line) NaCl fractions on a 1.5 × 115 cm column of CPG 10-240 glass beads using 0.5 M $CaCl_2$ as the eluting solvent. Four-milliliter fractions were collected and 50-μL aliquots analyzed for radioactivity. The peak elution positions of marker saccharides (HA, vitreous humor hyaluronic acid; C4S, porcine rib cartilage chondroitin 4-sulfate; GlcUA, glucuronic acid) are indicated by arrows. Fractions 26–31 (M0.2a), 32–42 (M0.2b), 26–33 (M0.4a), and 34–44 (M0.4b) were combined, dialyzed against water, and lyophilized.

0.03 M in NaCl and 0.15% in CPC. After 16–48 h at room temperature, the precipitate was collected by centrifugation (15 000*g*, 30 min) and washed three times with 0.15% CPC in 0.03 M NaCl by resuspension and centrifugation. In some experiments, further CPC was added to the supernatant and washes to a final concentration of 1%, and the small amount of precipitate formed was collected and washed as above. The precipitates were combined and fractionally extracted with 0.2, 0.4, 0.8, 1.2, and 2.0 M NaCl utilizing 1 × 20 mL and 3 × 10 mL for each extraction. The CPC in the extracts was removed by dialysis at 40–45 °C against 2 M NaCl followed by distilled water at 4 °C; the solutions were filtered and lyophilized (Table I).

The CPC in the combined supernatant and washings was precipitated by addition of KCNS. The precipitate was removed by centrifugation and washed with water, and the filtrate and washings were extensively dialyzed against distilled water. Lyophilization of the dialyzed solution gave a mixture of glycopeptides (class II). In the case of fractions from media, the mixture also consisted of a large proportion of glycopeptides arising from fetal calf serum.

Treatment of Cells with Tos-PheCH$_2$Cl-Trypsin. For the isolation of cell-surface complex saccharides, cells were cultured for 48 h prior to harvest in medium containing one third the usual amount of glucose and 0.5 μCi per mL of D-[1-^{14}C]glucosamine hydrochloride (New England Nuclear, 51.5 mCi/mmol). The cells were harvested and washed with balanced salt solution three times by resuspension and centrifugation and subjected to short-term successive incubations with Tos-PheCH$_2$Cl-trypsin (Codington et al., 1972). In preliminary experiments with unlabeled cells, it was noticed that considerable cell death and clumping occurred after the third incubation. In subsequent experiments, DNase (Sigma, electrophoretically purified) was included at 10 μg per mL of incubation mixture in order to prevent the clumping. Control incubations were done simultaneously with DNase and balanced salt solution. After each incubation period, the mixture was centrifuged (200*g*, 10 min) and the supernatant collected and cells resuspended in fresh solution and incubation repeated. All incubations were carried out in the cold room (4 °C) on a rotary table (100 rpm) for 20 min.

Agglutination of Cells by Plant Lectins. Cells were harvested by pouring off the media, washing the cell layer with calcium magnesium free phosphate buffered saline three times, and treating with 0.01 M ethylenediaminetetraacetic acid in the same buffer at 37 °C for 5–15 min. The cells were pelleted and washed with balanced salt solution three times by resuspension and centrifugation and finally suspended at 2 × 10^6 cells per mL in phosphate-buffered saline, pH 7.2. Lectin solutions also in phosphate-buffered saline were serially diluted starting at a concentration of 50 μg/mL. This solution (0.1 mL/well) in a plastic agglutination plate was incubated with 0.1 mL of the above cell suspension for 20 min at room temperature (20–25 °C) on a rotary table at 70 rpm. Controls without lectins and with lectins plus inhibitor saccharides (*N*-acetyl-D-glucosamine for WGA, methyl α-D-mannoside for Con A and lactose for RCAI) were included in order to check the specificity of the agglutinins. The agglutination was scored as: +++, heavily clumped; ++, moderately clumped; +, slightly clumped; −, not clumped.

Results

Table I presents the results of NaCl fractionation of the CPC precipitate. Fractions M0.8 to M2.0 contain glycosaminoglycans which have been characterized (Satoh et al., 1974; Bhavanandan and Davidson, 1977).

Sialoglycopeptides in M0.2 and M0.4—Class I Sialoglycopeptides. Chromatography of M0.2 and M0.4 on a CPG column produced the pattern illustrated in Figure 1. M0.2a, M0.4a, M0.2b, and M0.4b were isolated by pooling the indicated fractions, dialyzing to remove the salts in the eluent and lyophilizing. M0.2a and M0.4a were identical to the major component of M0.8 which was identified as a high-molecular-weight chondroitin (Bhavanandan and Davidson, 1977). M0.2b and M0.4b consisted of similar glycopeptides, referred to in the rest of the text as class I glycopeptides. Chromatography of class I glycopeptides (M0.2b) on CPG and Sephadex G-50 columns (Bhavanandan and Davidson, 1976) indicated that this fraction is relatively homogeneous on the basis of size. However, cellulose acetate electrophoresis in two different buffer systems showed two Alcian blue positive components, only one of which was labeled. This indicated that the labeled glycopeptide was contaminated by an acidic nonlabeled component apparently derived from serum. Further purification of the labeled component was achieved by chromatography on a DEAE-cellulose column using a linear gradient of 0.01 to 1.0 M pyridine acetate (Figure 2). The single peak obtained was isolated by preparative chromatography. Cellulose acetate electrophoresis in two buffer systems of the purified material is illustrated in Figure 3. The labeled glycopeptide was coincident with the major Alcian blue staining spot in both buffer systems; a trace of the unlabeled contami-

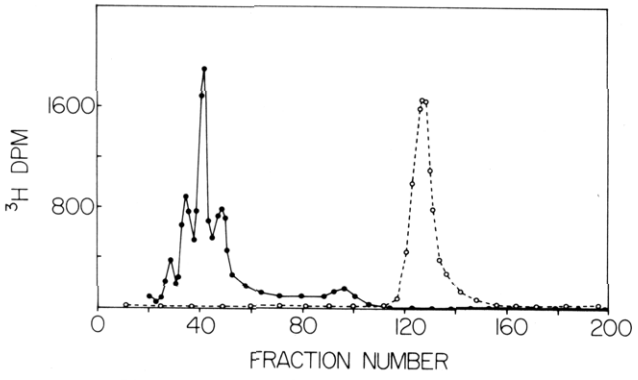


FIGURE 2: Chromatography of class I and II sialoglycopeptides on a DEAE-cellulose column (0.9 × 70 cm). Elution was with a linear gradient of 0.01 to 1.0 M pyridine acetate buffer, pH 5.1. Rate of flow was 30 mL/h. Fractions of 2.5 mL were collected and aliquots analyzed for radioactivity. Classes I (○) and II (●) were analyzed on separate runs and the results are presented by superimposing the elution patterns.

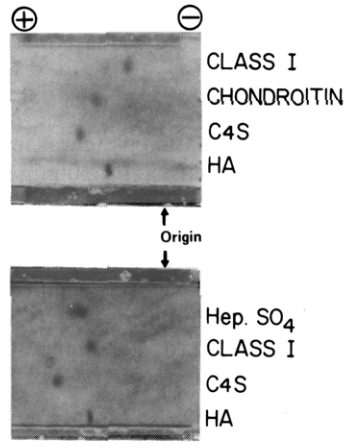


FIGURE 3: Cellulose acetate electrophoresis of class I sialoglycopeptide. *Upper*, in 0.2 M calcium acetate, 5 mA, 3 h; *lower*, in 0.1 N pyridine formate, pH 3.0; 10 mA, 20 min. Strips were stained with Alcian blue. Marker saccharides used were HA and C4S (see Figure 1), Hep·SO_4, heparan sulfate, and mouse melanoma chondroitin.

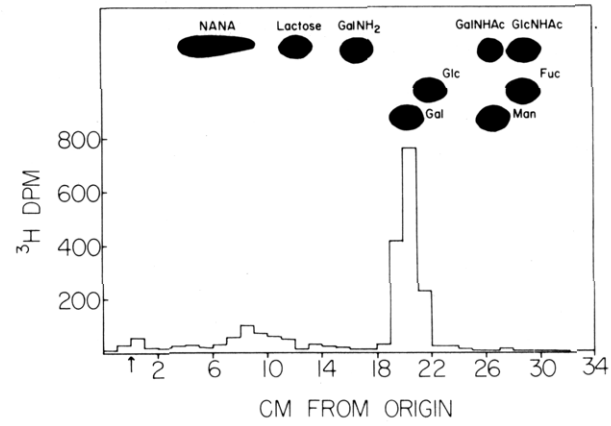


FIGURE 4: Paper chromatography of ^{3}H-labeled neutral sugars isolated from class I sialoglycopeptides in solvent system B. Standard saccharides were visualized with a silver nitrate stain.

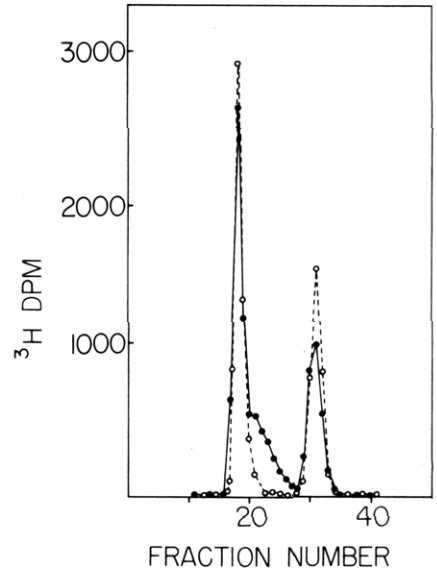


FIGURE 5: Gel filtration of class I sialoglycopeptide on a Bio-Gel P2 column (0.9 × 70 cm) after treatment with *Vibrio cholerae* neuraminidase (○) or treatment with neuraminidase followed by pronase (●). The low-molecule-weight material with peak position at fraction 31 had an elution profile identical to that of NANA.

nant was still evident in one buffer system, 0.2 M calcium acetate.

Sialic Acid and Hexosamine Analysis. The distribution of ^{3}H counts in sialic acid (38%) and hexosamine (62%) in class I glycopeptides and identification of these sugars as *N*-acetylneuraminic acid and *N*-acetylgalactosamine has been published (Bhavanandan and Davidson, 1976).

Neutral Sugars in Class I Glycopeptides. Class I glycopeptides isolated from cells and spent media obtained by growing the cells in the presence of [^{3}H]glucose were used in this experiment. The fractions obtained by 0.2 M NaCl extraction of the CPC precipitate were extracted with $CHCl_3$–CH_3OH (2:1) and the component insoluble in the organic solvent was purified by chromatography on CPG and a WGA–Sepharose 4B column to yield class I glycopeptides.

To determine the neutral sugar content, the glycopeptides were hydrolyzed (1 N HCl, 100 °C, 6 h) and after drying in a vacuum desiccator over NaOH and P_2O_5 the residues were dissolved in water and passed through AG50 (H^+) and AGI (formate) columns. The water eluates of the columns were lyophilized and examined by paper chromatography (Figure 4). The AG50 column was eluted with 1 N HCl, and the eluate was dried and hydrolyzed with 6 N HCl and examined on the amino acid analyzer by the stream-splitting technique employing ^{14}C-labeled glucosamine and galactosamine as internal standards. In addition to [^{3}H]galactosamine, the following ^{3}H-labeled amino acids were detected: glycine, alanine (the label corresponding to these two amino acids was not completely resolved), serine, aspartic acid, glutamic acid, proline, valine, and leucine, in this order of abundance.

Neuraminidase Susceptibility of Sialic Acid in Class I Glycopeptides. Vibrio cholerae neuraminidase released 50.9% sialic acid in 30 min, 84.5% in 3 h, and 97.4% after 24 h, whereas with influenza virus neuraminidase about 48.6% had been released in 1 h and 59.1% after 2 h with no further release up to 24 h. The labeled sialic acid released by acid hydrolysis was taken as 100%. The radioactive component released by *Vibrio cholerae* enzyme was identified as *N*-acetylneuraminic acid by paper chromatography and cochromatography on a Bio-Gel P2 column.

Retreatment of the asialo class I glycopeptides with pronase did not cause appreciable degradation of the molecule as illustrated in Figure 5.

Treatment of Sialo and Asialo Class I Glycopeptides with Various Glycosidases. The glycopeptides and the asialo glycopeptide prepared by treatment with *Vibrio cholerae* neu-

TABLE II: Action of Glycosidases on Sialo and Asialo Class I Glycopeptides.

Treatment	% Tritium-labeled components released: Sialoglycopeptide	Asialoglycopeptide	Remarks
β-Hexosaminidase (*Ch. lampus; D. pneumoniae*)	Not determined	0 (*Ch. lampus*), 3.6 (*D. pneumoniae*)	*D. pneumoniae* hexosaminidase product eluted on a Bio-Gel P2 column at the same position as Gal 1→[^{3}H]Gal*N*Ac
α-*N*-Acetylgalactosaminidase (*Ch. lampus*)	0	0	
β-Galactosidase (Jack bean) followed by β-hexosaminidase (*D. pneumoniae*)	Not determined	0	
β-Galactosidase (Jack bean) followed by α-*N*-acetylgalactosaminidase (*Ch. lampus*)	2 (Jack bean β-galactosidase)	27 (Jack bean β-galactosidase)	Product from asialoglycopeptide was identified as *N*-acetyl[^{3}H]galactosamine
Mixture of glycosidases (*Ch. lampus*)	Not determined	48	Product identified as *N*-acetyl[^{3}H]-galactosamine
endo-α-*N*-Acetylgalactosaminidase (*D. pneumoniae*)	0	80	Product from asialoglycopeptide was identified as Gal 1→[^{3}H]GalNAc

raminidase or mild acid (0.1 N H_2SO_4) followed by fractionation on a Bio-Gel P2 column were digested with various glycosidases singly or in combinations. The incubation mixtures were analyzed for release of ^{3}H-labeled components by gel filtration on a Bio-Gel P2 column. The results are summarized in Table II. When a labeled component was released, it was characterized by chromatographic techniques.

The labeled product from digestion with *endo*-α-*N*-acetylgalactosaminidase was isolated by chromatography on a Bio-Gel P2 column and identified as Gal 1(β)→[^{3}H]Gal*N*Ac by paper chromatography (Figure 6). The disaccharide migrates with a mobility close to that of $GalNH_2$, whereas after treatment with Jack bean β-galactosidase the labeled product has a mobility identical to that of Gal*N*Ac. The identity of this disaccharide was further confirmed by reduction with NaB^3H_4 followed by acid hydrolysis and detection of [^{3}H]galactosaminitol on the amino acid analyzer using the citrate–borate buffer system (Bella and Kim, 1970). However, only 38% of the label in the hydrolysate eluted with galactosaminitol. A large portion of the balance eluted at the beginning of the chromatogram before aspartic acid; this arises from an acid-resistant contaminant in NaB^3H_4 (McLean et al., 1973). Some labeled material eluted after galactosamine and is apparently due to reduced chromogen derived from terminal *N*-acetylgalactosamine (Bray et al., 1967) (cf. results in Figure 10).

Action of Alkaline Borohydride on Class I Glycopeptides. The elution profile of the glycopeptide on a CPG column before and after treatment with alkaline borohydride showed clear evidence of complete alkali elimination. The gel-filtration profiles on Bio-Gel P4 and P6 columns of the products of alkaline borohydride treatment of class I glycopeptides together with markers including tetra- and trisaccharides obtained by similar treatment of fetuin are illustrated in Figure 7. The labeled material eluting in peaks I (25–31) and II (32–37) on the Bio-Gel P4 column was recovered by lyophilization and treated three times with methanol to remove borate. Paper chromatography in solvent systems A (Figure 8) and C showed that the major labeled components in peaks I and II had mobilities similar to the tetra- and trisaccharides obtained by treatment of fetuin with alkaline borohydride. In both solvents other minor unidentified spots were detected. The major peak I (47–52) from the Bio-Gel P6 column gave a single sharp spot coincident with fetuin tetrasaccharide when examined by paper chromatography using solvent system C.

Hydrolysis of the β-elimination products of class I glycopeptides followed by stream-split analysis on the amino acid analyzer employing the citrate–borate system gave only

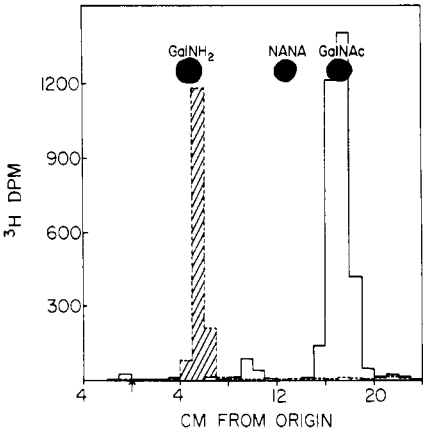


FIGURE 6: Paper chromatography of the low-molecular-weight material obtained by digesting asialo class I glycopeptide with *endo*-α-*N*-acetylgalactosaminidase before (shaded) and after (unshaded) treatment with Jack bean β-galactosidase. The chromatogram was developed in solvent system A and the standard sugars were detected with silver nitrate reagent.

[^{3}H]galactosaminitol with no detectable [^{3}H]galactosamine.

The distribution of label in the tetra- and trisaccharides from class I glycopeptide (peaks I and II, respectively, from the Bio-Gel P4 column) is 73 and 27%. If it is assumed that the specific activity of the three (two from tetra and one from tri) residues of sialic acid are the same and also the specific activities of two residues of GalNAc are the same, then the ratio of tetra- to trisaccharide is 3.24, since 38% of the label in the class I glycopeptides is in sialic acid.

The class I glycopeptide isolated from cells grown in the presence of [^{3}H]glucose was treated with alkaline borohydride, neutralized with acetic acid, and passed through an AG50 (H^+) column. The column was washed with water and the β-eliminated oligosaccharides were recovered by lyophilization and subjected to periodate oxidation (Spiro and Bhoyroo, 1974). Periodate-oxidized sodium borohydride reduced products were neutralized with HCl and passed through coupled AG50 (H^+) and AGI (formate) columns. The sialylated oligosaccharides were eluted from the AGI column, concentrated to dryness, and hydrolyzed with 4 N HCl, 100 °C for 4 h. The hydrolysis products, together with the products from a control sample treated similarly but with destroyed periodate, were examined on paper chromatograms (Figure 9).

In order to determine the amino acids involved in the *O*-

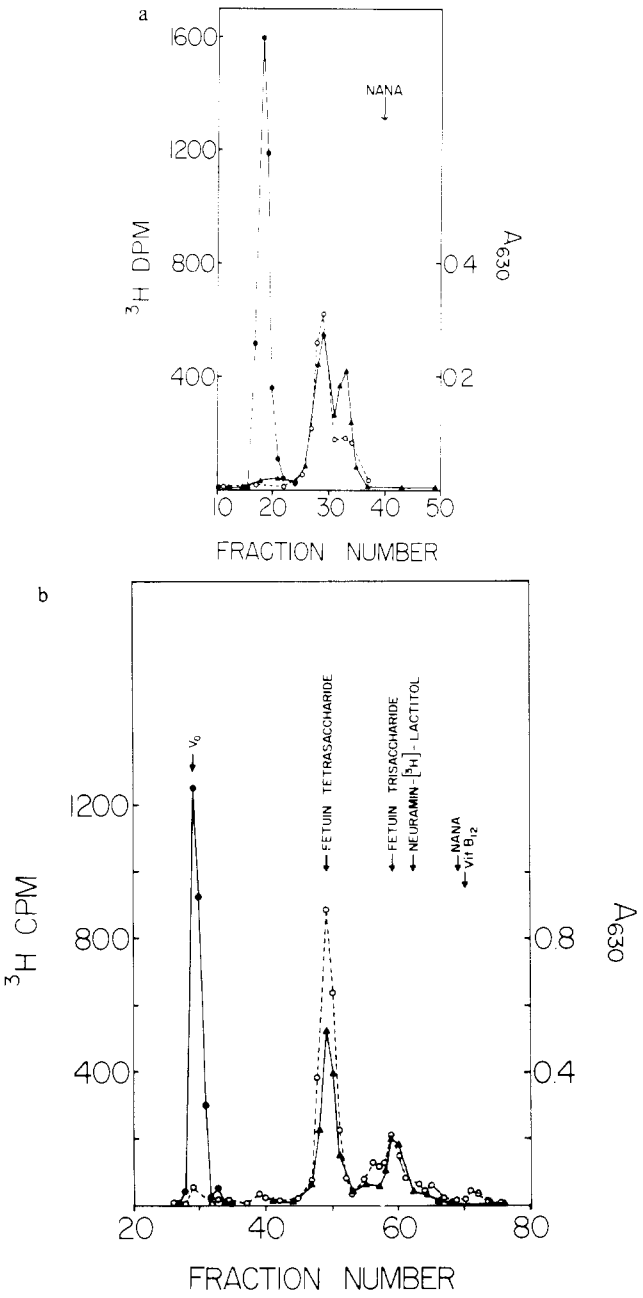


FIGURE 7: Gel filtration of class I sialoglycopeptides before (●) and after (○) treatment with alkaline borohydride on a (a, top) Bio-Gel P4 column (0.9 × 70 cm) and a (b, bottom) Bio-Gel P6 column (0.9 × 104 cm). The treated samples were mixed with tetrasaccharides and trisaccharides isolated from alkaline borohydride treatment of fetuin prior to chromatography. The elution pattern (▲) of these oligosaccharides was detected by analyzing aliquots of the fractions for sialic acid (Jourdian et al., 1971). The peak elution of blue dextran (V_0) and other standards on the same columns are indicated by arrows.

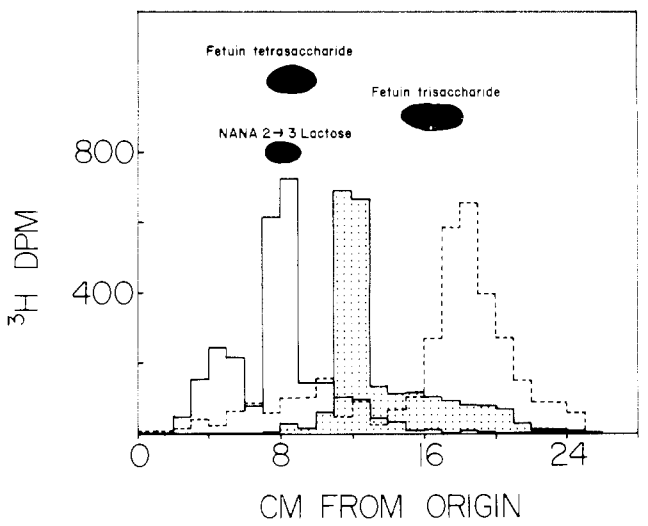


FIGURE 8: Paper chromatography of β-eliminated oligosaccharides from class I glycopeptides isolated by Bio-Gel P4 fractionation; peak 25–31, solid line; and peak 32–37, broken line. Chromatography was in solvent system A. The dotted area shows the mobility of neuramin[^{3}H]lactitol. Positions of other standards were detected by periodate–benzidine staining.

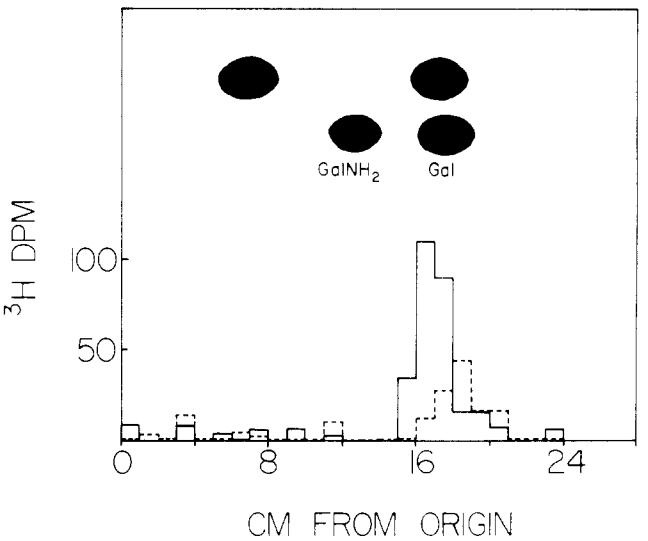


FIGURE 9: Paper chromatography of acid hydrolysates of sialyl oligosaccharides from class I glycopeptides before (broken line) and after (solid line) periodate oxidation. Chromatography was in solvent system D. The standards ($GalNH_2$ and Gal) and the products obtained by similar treatment of fetuin trisaccharide (unmarked spots) were visualized by the periodate–benzidine reagent.

glycosidic linkage to *N*-acetylgalactosamine, the class I glycopeptide was treated with NaB^3H_4. The NaB^3H_4 (New England Nuclear specific activity 276 mCi/mmol) was dissolved in 100 μL of 0.1 N NaOH to give a solution of 0.9 M NaB^3H_4 concentration. The sample was dissolved in 20 μL of the above NaB^3H_4 and incubated for 48 h at 37 °C in the dark under an N_2 atmosphere. A solution of 1 M $NaBH_4$–0.1 N NaOH (100 μL) was added and the incubation continued for a further 24 h under the same conditions. Acetone was added to destroy NaB^3H_4 and the 2-[^{3}H]propanol distilled off. The residue was repeatedly dissolved in water and lyophilized to remove exchangeable tritium. An aliquot was hydrolyzed (6 N HCl, in vacuo sealed tube at 110 °C for 20 h) and the hydrolysate, after drying, analyzed by the stream-splitting technique on the amino acid analyzer using labeled and unlabeled standards. A control using 20 μL of the same solution NaB^3H_4 was treated identically. The results are illustrated in Figure 10. The tritium activities derived from the treated sample correspond to alanine (fractions 56–58), α-amino butyric acid (fractions 64–67), galactosaminitol (114–120) which elutes in the same position as galactosamine in the buffer system used, and an unknown (fractions 126–133) which is apparently the reduced Kuhn's chromogen (Bray et al., 1967). Tritium activity eluting in the position of glycine was detected both in the sample and in the NaB^3H_4 control. The control also had large quantities of label eluting before aspartic acid. In another experiment,

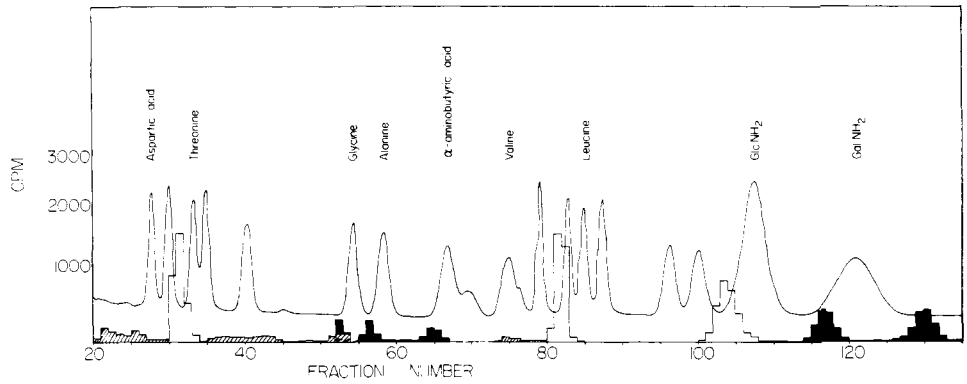


FIGURE 10: Analysis on the amino acid analyzer using a stream-splitting technique of the 6 N HCl hydrolysate of alkaline borotritide (B^3H_4) treated class I glycopeptides. The elution of tritium activity from the sample is shown by the black area and from the NaB^3H_4 control by the shaded area. The radioactivities of internal standards [^{14}C]threonine, [^{14}C]leucine, and [^{14}C]glucosamine are shown by the unshaded areas.

the product from NaB^3H_4 treatment was applied on the AG50 (H^+) column and, after washing with deionized water, the peptides were eluted with 1 N NH_4OH. The NH_3 in the eluate was removed by a stream of N_2 and the solution lyophilized to yield the ^{3}H-labeled peptides. Hydrolysis followed by amino acid analysis gave results similar to those above.

Affinity Chromatography of Class I Glycopeptides on Lectin–Sepharose Columns. The class I sialoglycopeptide and the asialoglycopeptide prepared by neuraminidase treatment were not retained on Con A–Sepharose. The activity of the Con A column was demonstrated by the binding of ovalbumin glycopeptides to the lectin and their elution with 0.1 M methyl α-mannoside solution.

The sialoglycopeptide, however, bound to a WGA–Sepharose column and was eluted with 0.1 M *N*-acetylglucosamine in buffer. Asialoglycopeptides prepared either by mild acid hydrolysis as well as partially (46%) or totally (100%) desialylated glycopeptide obtained by *Vibrio cholerae* neuraminidase treatment were not adsorbed on this lectin column, whereas a preparation desialylated to 16% was retained on the WGA column. In order to determine whether the retention of the sialoglycopeptide on the WGA–Sepharose column was due to an anion-exchange effect, the following experiment was done. The sialoglycopeptide was applied to the lectin column and after washing with buffer the column was eluted with 0.05 M NaCl in buffer prior to elution with 0.1 M *N*-acetylglucosamine. The results, illustrated in Figure 11, show that only a trace of the glycopeptide eluted with 0.05 M NaCl, whereas the major portion was displaced only with the sugar. A sample of ^{3}H-labeled hyaluronic acid applied on the WGA–Sepharose column was not retained. The oligosaccharides prepared by β elimination from the glycopeptide were not retained on the column, illustrating that the carbohydrates must be attached to the peptide core for interaction with WGA.

The asialoglycopeptide bound to RCAII–Sepharose columns and was specifically eluted with 0.1 M lactose; it also bound partially to the RCAI-Sepharose column, about 13–33% in different experiments. The sialoglycopeptide did not bind to either RCAI- or RCAII–Sepharose columns.

Molecular Weight Determination of Class I Sialoglycopeptide. The class I sialoglycopeptide (1.84 mg) isolated from media was further purified as follows. It was dissolved in 50 mM Tris-HCl buffer, pH 7.6, containing 10 mM Mg^{2+} and treated with deoxyribonuclease I (Sigma DN-EP) and ribonuclease (Boehringer) at 37 °C for 48 h. The sample was recovered by dialysis and freeze-drying. It was then dissolved in 0.1 M NaOAc, pH 5.0, containing 0.15 M NaCl and digested with bovine testicular hyaluronidase for 40 h. The digest

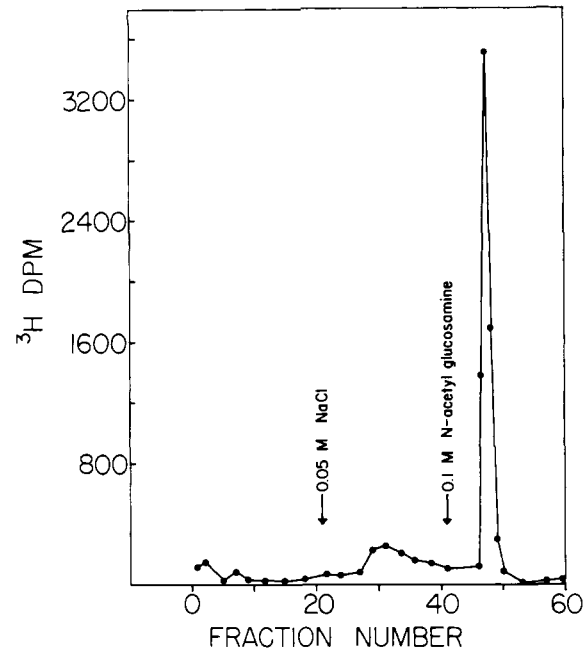


FIGURE 11: Affinity chromatography of class I sialoglycopeptides on a WGA–Sepharose column. Elution was with 0.05 M Tris-HCl, pH 8.0, followed by 0.05 M NaCl in buffer, and finally *N*-acetylglucosamine. One milliliter fractions were collected and analyzed for radioactivity.

was dialyzed against Tris-HCl buffer, pH 7.6, containing 10 mM Ca^{2+} and treated with pronase at 40 °C for 3 days in the presence of toluene; enzyme was added at 0, 24, and 48 h. The treated sample was purified by chromatography first on a CPG column and then on a DEAE-cellulose column. The labeled material (1.2 mg) was recovered and used for sedimentation equilibrium analysis and for gas–liquid chromatography.

The molecular weights of this purified material determined by sedimentation equilibrium analysis as described previously (Bhavanandan and Davidson, 1977) were 6600 and 8800 when run at two different speeds. A partial specific volume ($\bar{v}$) of 0.66 was assumed in the calculation of molecular weights. Codington et al. (1972) calculated the $\bar{v}$ of epiglycanin, a mucin-type glycoprotein from TA_3Ha cells, to be 0.66.

Gas–Liquid Chromatographic Analysis of Class I Glycopeptides. The purified class I glycopeptides were methanolyzed and analyzed by gas–liquid chromatography as described (Clamp et al., 1971), except that re-N-acetylation was done in the presence of pyridine instead of silver carbonate (Etchison and Holland, 1975). The analysis showed the presence of ga-

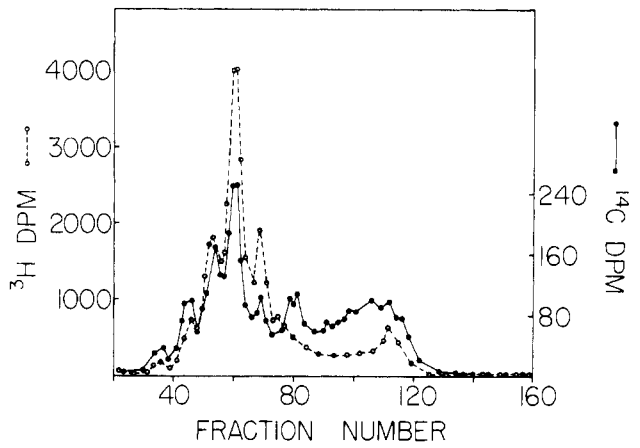


FIGURE 12: Cochromatography of ^{14}C-labeled Tos-PheCH$_2$Cl-trypsinate components (●) and class II sialoglycopeptides (○) on a DEAE-cellulose column. Elution was with a linear gradient of 0.01 M pyridine acetate (300 mL) to 1.0 M pyridine acetate (300 mL). Fractions of 3.5 mL were collected and 1-mL aliquots assayed for radioactivity.

lactose, *N*-acetylgalactosamine, and sialic acid in the ratio of 1.0:0.86:1.63. Another sample which was purified on a DEAE-cellulose column but not digested with nucleases and hyaluronidase showed *N*-acetylglucosamine in addition to the above sugars in the ratio of 1.0:0.71:2.0:1.04 (GlcNAc). In addition to these sugars glucose was also detected (see Discussion).

Class II Glycopeptide (Glycopeptides not Precipitated with CPC). The glycopeptides were isolated from the CPC supernatant of the pronase digest of spent media and cells obtained by cultivation of cells in the presence of [^{3}H]glucosamine (B16 media). A representative distribution of the radioactivity in sialic acid and hexosamine is sialic acid (25%), glucosamine (66%), and galactosamine (9%). Gel filtration on CPG and Sephadex G-50 columns indicated that these glycopeptides were smaller in molecular weight than the class I glycopeptides (Bhavanandan and Davidson, 1976). Chromatography on DEAE-cellulose column (Figure 2) resulted in resolution into a number of components. The characterization of these glycopeptides will be reported in a subsequent publication.

Agglutination of B16 Melanoma Cells by Plant Lectins. The results of agglutination experiments on B16 mouse melanoma cells using WGA, Con A, and RCAI are given in Table III. All three lectins agglutinated these cells strongly, whereas soybean agglutinin was inactive even at a concentration of 500 μg/mL. Treatment of the cells with *Vibrio cholerae* neuraminidase for 30 min rendered them agglutinable by soybean lectin. The agglutination by Con A was inhibited by methyl α-D-mannoside and that by RCAI by lactose but not by *N*-acetyl-D-galactosamine. Both *N*-acetyl-D-glucosamine and *N*-acetylneuraminic acid inhibited agglutination by wheat germ lectin, the former at 40 mM and latter at 80 mM. Class I glycopeptides purified by DEAE-cellulose column chromatography also inhibited the wheat germ induced agglutination of melanoma cells at a concentration of 74 μg/mL, 0.1 mM or less.

Isolation of Surface Components from ^{14}C-Labeled Cells. The results of a typical experiment are given in Table IV. The release of considerable ^{14}C-labeled material in the absence of trypsin was unexpected. The supernatants from the first three trypsin incubations were combined for further investigations.

After centrifugation at 15 000 rpm for 20 min to remove any cell debris, the supernatant was dialyzed against deionized water and lyophilized. The lyophilized material was dissolved in 0.5 mL of 50 mM Tris-HCl buffer, pH 8.0, containing 0.01 M Ca^{2+} and incubated at 37 °C for 60 h with pronase (0.5 mg at time 0, 20, and 40 h) in the presence of toluene. The mixture was dialyzed against deionized water and lyophilized to yield [^{14}C]glucosamine-labeled complex saccharides.

Cochromatography of Tos-PheCH$_2$Cl-trypsinization Products and Media Glycopeptides. The [^{14}C]glucosamine-labeled material obtained by pronase treatment of Tos-PheCH$_2$Cl-trypsinate was chromatographed together with [^{3}H]glucosamine-labeled class II glycopeptides obtained from media on CPG, Sephadex G-50, and DEAE-cellulose columns. The elution profiles of both isotopes from each of these columns were remarkably similar. The profile on the DEAE-cellulose column, which was the most complex, is illustrated in Figure 12. The similarity of the profiles is striking up to about fraction 75. The difference in the pattern beyond this fraction may be at least partly due to the fact that Tos-PheCH$_2$Cl-trypsinate was not treated with CPC and would thus include the glycosaminoglycans which are absent in ^{3}H-labeled class II glycopeptides isolated from media. Chromatography of pronase digested Tos-PheCH$_2$Cl-trypsinate on a WGA–Sepharose column showed the presence of class I glycopeptide in this material.

TABLE III: Agglutination of B16C3 Mouse Melanoma Cells by Wheat Germ, Con A, and *Ricinus communis* I Lectins.[a]

Lectin concn (μg/mL)	Deg of agglutination		
	WGA	Con A	RCAI
50	+++	+++	+++
25	+++	+++	+++
12.5	+++	+++	++(+)
6.25	++	++	++
3.13	+	++	++
1.56	(+)	+(+)	+
0.78	−	+	(+)
0.39	−	+	−
0.19	−	(+)	−

[a] See Experimental Procedure for details about scoring of agglutination.

TABLE IV: Complex Saccharide Release on Successive Incubation of [^{14}C]Glucosamine-Labeled B16 Melanoma Cells with Tos-PheCH$_2$Cl-trypsin.[a]

Treatment	Incubation	% Viable cells	^{14}C dpm released per 10^6 cells
Tos-PheCH$_2$Cl-trypsin[b] and DNase[c]	0	92	
	1	92	15 443
	2	91	10 275
	3	91	7 928
	4	87	9 738
	5	82	6 963
DNase[c]	0	92	
	1	91	4 969
	2	90	4 628
	3	88	2 925
	4	83	4 259
	5	83	3 105

[a] All incubations were at 4 °C for 20 min. [b] 20 μg/mL in balanced salt solution. [c] 10 μg/mL in balanced salt solution.

Discussion

It is evident from the results of the present investigation that cultured B16 mouse melanoma cells synthesize several gly-

coproteins which can be detected both at the cell surface as well as in the growth medium. The nondialyzable glycopeptides derived by pronase digestion of the cells or spent media were fractionated into two groups on the basis of their interaction with CPC. Sialoglycopeptides which apparently formed a weak complex with CPC were selectively eluted from the precipitate with 0.2–0.4 M NaCl, whereas the complexes between the strongly anionic glycosaminoglycans and CPC required higher concentrations of NaCl for dissociation. These class I sialoglycopeptides which constitute about 4–6% of the nondialyzable ^3H activity incorporated into the complex saccharides were purified to apparent homogeneity by gel filtration on CPG columns and by anion-exchange chromatography on DEAE-cellulose columns. A trace amount of a serum-derived nonlabeled component staining with Alcian blue was still detectable by cellulose acetate electrophoresis (Figure 3). The labeled sugar components were NANA and *N*-acetylgalactosamine. The identity of the former was based on cochromatography with standard on a Bio-Gel P2 column, by paper chromatography, and paper electrophoresis. The galactosamine was identified on the amino acid analyzer and by ninhydrin oxidation followed by paper chromatography.

The neutral sugar (galactose) was identified using cells grown in the presence of [^3H]glucose. The isotope yield of the class I glycopeptide in the experiment was poor but this was expected due to the incorporation of glucose into various metabolic components. However, the neutral sugars isolated from purified class I glycopeptide on paper chromatography showed only [^3H]galactose; some slower moving labeled material apparently represents oligosaccharides arising from incomplete hydrolysis. Again, the only hexosamine obtained from those glycopeptides was [^3H]galactosamine; several labeled amino acids were also detected.

Analysis by gas–liquid chromatography of the class I glycopeptide isolated from spent media purified on a DEAE-cellulose column showed galactose, *N*-acetylgalactosamine, sialic acid, glucose, and *N*-acetylglucosamine. The first three sugars were expected on the basis of the analysis of metabolic labeled products; the presence of the latter two sugars suggested contamination by unlabeled components. *N*-acetylglucosamine was not detected in a sample which was pretreated with hyaluronidase; thus, a hyaluronic acid contaminant could be responsible for this sugar. Glucose was still present and would be due to glucose containing macromolecular contaminants from DEAE-cellulose or Sephadex G-50 columns, or dialysis tubings (Codington et al., 1972).

The action of neuraminidase on the purified glycopeptide showed that all sialic acid was present as terminal residues. However, the differential action of *Vibrio cholerae* and influenza virus neuraminidases suggested that this sugar may be involved in more than one type of linkage (Bhavanandan and Davidson, 1976). Information on the sequence of the other sugars was obtained by treatment of the asialoglycopeptide with various glycosidases (Table II). The only treatment which released labeled monosaccharide, identified as *N*-[^3H]-acetylgalactosamine, was the combination of Jack bean β-galactosidase and α-*N*-acetylgalactosaminidase. This suggested the presence of the sequence $(Gal)_x(\beta)\rightarrow$[$^3H$]Gal*N*Ac($\alpha$)$\rightarrow$ in the asialo class I glycopeptide. This was confirmed by the release of the disaccharide Gal 1(β)$\rightarrow$[^3H]-Gal*N*Ac on digesting the asialo glycopeptide with *endo*-α-*N*-acetylgalactosaminidase. The disaccharide was released in a yield of about 80% by the endo enzyme.

Treatment of the glycopeptides with alkali in the presence of borohydride resulted in complete degradation to low-molecular-weight labeled components. The detection of only [^3H]galactosaminitol after hydrolysis of the β-elimination products indicated that there was only one galactosamine per prosthetic group which was involved in an alkali-labile linkage to the peptide. This together with the isolation of the disaccharide Gal$\rightarrow$Gal*N*Ac from *endo*-galactosaminidase treatment of asialo class I glycopeptides suggested that the glycopeptide had a mucin-type structure in which several oligosaccharides of the type $NANA_x$[Gal $\rightarrow$ Gal*N*Ac] were attached to the protein. The two major alkaline borohydride degradation products of this glycopeptide and that of fetuin (Spiro and Bhoyroo, 1974) cochromatographed on Bio-Gel P2, P4, and P6 columns and on paper in two different solvent systems. This together with the similarity of the composition of the oligosaccharides from the two sources suggests that the melanoma class I glycopeptides may have prosthetic groups similar, if not identical, to those found in fetuin. The identity of the two other oligosaccharide peaks (minor components) detected on the Bio-Gel P6 column was not investigated further due to insufficient material. The detection of [^3H]galactose after periodate oxidation of β-eliminated oligosaccharides suggests that position 3 of galactose is substituted. Preliminary results from periodate oxidation of the native class I sialoglycopeptide indicate that the Gal*N*Ac is also substituted on position 3. Confirmatory evidence for the linkages in these oligosaccharides will have to be obtained by methylation analysis; this will be feasible using glycopeptides isolated from solid tumor.

Evidence for the involvement of both serine and threonine in *O*-glycosidic linkages to *N*-acetylgalactosamine was obtained by the detection of both [^3H]alanine and α-[^3H]aminobutyric acid after β elimination in the presence of NaB^3H_4. Even though the ratio of the two amino acids was 8:7, this may not reflect the relative proportion of serine and threonine residues involved in the *O*-glycosidic linkage, since the unsaturated amino acids obtained by elimination (dehydroalanine and α-aminocrotonic acid) are not reduced by $NaBH_4$ to the same extent (Tanaka and Pigman, 1965).

It is thus apparent that oligosaccharide units, mostly tetrasaccharides [NANA$\rightarrow$3Gal$\rightarrow$(NANA$\rightarrow$(?))3Gal*N*Ac] and some trisaccharides, are attached *O*-glycosidically to threonine and serine in the class I glycopeptide. The presence of trisaccharides may reflect incomplete sialylation during biosynthesis or enzymatic or chemical breakdown during isolation. The molecular weight of this glycopeptide was estimated to be about 12 000–15 000 on the basis of its mobility on the CPG column, since it had an elution profile identical to that of cartilage chondroitin 4-sulfate chains. Equilibrium sedimentation analysis suggested a value in the range of 6500 to 9000. The estimate obtained by gel filtration using a polyanionic polysaccharide as reference is probably more reliable than the results from sedimentation analysis. Low-molecular-weight unlabeled contaminants will lower the estimated molecular weight of the sialoglycopeptide; further, the partial specific volume (0.66) used in the calculation is an assumption (see Results). In either case the apparent molecular weight of the pronase-resistant glycopeptide seems high. Since the molecular weight of the tetra- and trisaccharide units would be about 1000 and 700, respectively, it may be estimated that 7–9 such units are present in the pronase core structure. This glycopeptide was resistant to further digestion by pronase. Even removal of sialic acid does not alter the susceptibility to pronase very much, since the asialoglycopeptide was only minimally degraded by this enzyme (Figure 5). This suggests that the saccharides may be clustered in groups on the peptide chain. In this aspect, as well as in the structure of the tetra- and trisaccharides, the class I glycopeptide of mouse melanoma has

an apparent similarity to the major glycoprotein of the erythrocyte membrane (Thomas and Winzler, 1969; Tomita and Marchesi, 1975). The structure of the trisaccharide is similar to the short-chain-type oligosaccharide of the TA_3-Ha cell surface glycoprotein (Codington et al., 1975). The glycopeptides containing *O*-glycosidically linked carbohydrate units obtained by pronase digestion of fetuin were found to be included in Sephadex G-50 and G-25 columns in agreement with the results of Spiro and Bhoyroo (1974) and, therefore, are of lower molecular weight than the class I glycopeptide which is excluded on a Sephadex G-50 column (Bhavanandan and Davidson, 1976). The absence of clustered *O*-glycosidically linked carbohydrate units in fetuin was advantageous in purifying the class I glycopeptide from the spent media containing fetal calf serum.

Affinity chromatography of glycopeptides on lectin columns of defined specificities might be expected to provide structural information on the molecule (Sharon and Lis, 1972) in addition to yielding data on the homogeneity of the preparations. Class I glycopeptide interacted with WGA as shown by the retention of the labeled component on a WGA–Sepharose column and its subsequent elution by *N*-acetylglucosamine (Figure 11). Since this fraction does not contain any labeled *N*-acetylglucosamine, the sugar generally believed to be the ligand for this lectin (Sharon and Lis, 1972), the observed affinity was probably due to sialic acid. This conclusion is consistent with the observations by other workers that WGA also has an affinity for sialic acid (Greenaway and LeVine, 1973) and sialoglycopeptides (Adair and Kornfeld, 1974). Confirmation of this hypothesis was obtained by the failure of the desialylated fraction to bind to WGA–Sepharose and by the inability of NaCl of the same ionic strength as of the *N*-acetylglucosamine solution to displace the bound sialoglycopeptide from the lectin column. The polyanion hyaluronic acid was not retained on this column, excluding an ion-exchange effect. Even though WGA is able to interact with sialic acid and sialoglycopeptides, it apparently binds only to those molecules with a high sialic acid content and having specific structural features. The class II sialoglycopeptides and the partially desialylated class I glycopeptides were not retained by the WGA–Sepharose column. Most interestingly, the oligosaccharides prepared from class I by β elimination had no affinity to the WGA–Sepharose 4B column, suggesting that several concurrent sialyl oligosaccharides attached to a peptide backbone are essential for the binding process. It is possible that the binding site for the sialic acid is the same as that for *N*-acetylglucosaminyl residues, or that it is conformationally affected by the hexosamine binding site. Sialoglycopeptides obtained from erythrocyte membrane also showed a similar interaction with WGA (Bhavanandan, unpublished results).

The sialoglycopeptide did not bind either to RCAI or RCAII–Sepharose columns, but the asialoglycopeptide bound to the latter completely. This is in agreement with the sequence Gal→Gal*N*Ac in the asialoglycopeptide (Bhavanandan, unpublished results).

In contrast to the class I sialoglycopeptide which formed a precipitate with CPC, the material which remained unprecipitated was a complex mixture of ^{3}H-labeled glycopeptides (class II). Preliminary evidence indicates that this class consists mostly of serum-type glycopeptides in which the carbohydrate units are linked to peptide through *N*-glycosidic linkages between *N*-acetylglucosamine and asparagine (unpublished results).

The agglutination studies using different lectins indicate that the B16 mouse melanoma cells have receptor sites on their surface for WGA, Con A, and RCAI lectins. The WGA agglutinability of the cells as well as the inhibition of this agglutination at very low concentration by class I glycopeptide seems to indicate that this large mucin-type sialoglycoprotein is located on the cell surface. In order to obtain further information on the relationship between the class I and II glycopeptides isolated from spent media and those present on the cell surface, we isolated the surface glycopeptides by mild treatment of [^{14}C]glucosamine-labeled cells with Tos-PheCH_2Cl-trypsin. It was observed that an unexpectedly large amount of labeled material was released by cells in the absence of trypsin, suggesting a natural rapid shedding or turnover of surface components. It is then conceivable that the use of exogenous proteolytic enzymes only accelerates a natural turnover of cell-surface components and that the molecules shed by the natural process, possibly nonproteolytic, would be larger in size than those released by exogenous proteolysis. In this context, it is interesting to note that Cooper et al. (1974) found appreciable molecular-size differences in the epiglycanin isolated by Tos-PheCH_2Cl-trypsin treatment of TA_3 cells and that shed by these cells in vivo into the ascites fluid and serum of host mice. The material released from B16 melanoma cells by mild Tos-PheCH_2Cl-trypsin treatment was digested with pronase and cochromatographed with ^{3}H-labeled fractions from media in several different systems. Marked similarity between the cell surface (^{14}C-labeled) and media (^{3}H-labeled) components was evident, suggesting that the class I and II sialoglycopeptides detected in the media have their origin at the cell surface. The actual nature of this association is difficult to elucidate. The comparison between the cell surface and media components mentioned above is based solely on the shape, size (CPG, Sephadex G-50), and charge (DEAE-cellulose); structural differences between these two classes of components cannot be excluded from our results.

We have demonstrated the ability of these cells to make class I and II sialoglycopeptides when grown in a chemically defined media containing 1% bovine serum albumin but devoid of serum (Banks et al., 1977). The class I sialoglycopeptides have also been isolated from solid melanoma tumors grown in syngeneic mice (V. S. Fareed et al., unpublished results). These findings rule out the possibility of the products described in this paper as being artifacts due to the in vitro culture system.

The synthesis and shedding (or secretion) of the class I (mucin-type) glycoprotein by mouse melanoma cells which are of neural origin are of interest. Evidence for the production and shedding in vitro and in vivo of mucin-type glycoproteins by other cancerous cells, particularly those grown in Ascites form, has been presented by several investigators (Adams, 1965; Langley and Ambrose, 1967; Molnar et al., 1965; Codington et al., 1972, 1975; Funakoshi et al., 1974). Human melanoma and breast cancer cells also produce galactosamine-rich sialoglycopeptides with affinity for WGA–Sepharose columns (V. P. Bhavanandan et al., unpublished results). In contrast, normal tissues, such as mouse iris melanocytes (Satoh et al., 1974), normal and regenerating rat liver (Akasaki et al., 1975), human fetal iris melanocytes (Banks et al., 1976), and a normal human mammary cell line (E. V. Chandrasekaran et al., unpublished results), either do not produce such or produce comparable glycoproteins in markedly reduced amounts. It is also of interest that the immediate precursor of blood group MN determinants, which is believed to have the structure Gal→Gal*N*Ac→Ser/Thr, has been detected in the cell membranes of breast cancers but not in healthy mammary glands or benign tumors by immunological methods (Springer et al., 1974). The reason for the capacity of malignant cells to synthesize and shed mucin-type sialoglycoproteins is not clear even though several hypotheses may be put forward. For ex-

ample, the mucin-type class I sialoglycoprotein of mouse melanoma could be involved in masking tumor antigens present on the cell surface as proposed for epiglycanin of TA_3Ha cells (Codington et al., 1973). Alternatively, the protein part of these glycoproteins is itself antigenic and the clustered negatively charged sialyl oligosaccharides function in masking the antigenic sites of the protein. The relationship between the melanoma-associated antigen (Bystryn et al., 1974) and the class I and II glycopeptides produced by the mouse melanoma is currently under investigation.

Acknowledgments

We express our appreciation to the following: Jeffrey Kemper, Anne Katlic, Debora Breindel, and Ellen Goldberg for technical assistance; Dr. Jan Tkacz for providing the first Con A-Sepharose and WGA-Sepharose preparations used in these studies; Mord Moore for amino acid and hexosamine analyses; and Dr. Y.-T. Li for Jack bean β-galactosidase.

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