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ISOLATION AND CHARACTERIZATION OF GLYCOCONJUGATES FROM B16 MOUSE MELANOMA TUMORS

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SUMMARY

The major glycosaminoglycans isolated from B16 mouse melanoma tumors after Pronase digestion were shown to be a family of chondroitin 4-sulfates with different degrees of sulfation and a wide molecular-weight range. Ultracentrifugation data gave molecular weight values as high as 88 000, in contrast to that of costal cartilage chondroitin 4-sulfate which is about 14 000. A mucin-type sialoglycopeptide, isolated from the tumors by cetylpyridinium chloride precipitation of the Pronase digest, was shown to contain *O*-glycosylically linked tetra- and tri-saccharides consisting of sialic acid, galactose, and *N*-acetylgalactosamine. The sialoglycoprotein, which on Pronase digestion gave rise to the glycopeptide, was isolated from the tumor by extraction with lithium diiodosalicylate and affinity chromatography on a wheat-germ agglutinin–Sepharose 4B column. It was homogeneous on the basis of gel filtration on Sepharose 4B and Sephadex G-200 columns, lectin affinity, and ion-exchange chromatography. The compounds isolated from the B16 mouse melanoma tumors are similar to those produced by the cultured melanoma cells, which suggests that the latter compounds are not artifacts of the culture system.

INTRODUCTION

The presence of glycosaminoglycans and glycoproteins in a variety of normal and cancerous cells has been described. In the case of B16 mouse melanoma cells grown in culture, the glycosaminoglycans and glycopeptides have been characterized in some detail: The glycosaminoglycans include a family of chondroitin 4-sulfates with unusually high molecular weights[1,2], and the mucin-type sialoglycopeptide isolated was shown to contain the structure $(NeuAc)_2 \rightarrow (Gal \rightarrow GalNAc)$ *O*-glycosylally linked to serine and threonine. This glycopeptide had the interesting characteristic of binding to wheat-germ agglutinin, a characteristic that was dependent on the proportion of terminal sialic acid groups present[3,4]. The present study was undertaken to provide a direct comparison between the macromolecules synthesized by tumor cells grown *in vitro* with those obtained from *in vivo* proliferating tissue. The presence of similar macromolecular, cell-surface components for both types of growth conditions

tends to strengthen the biological applicability of results obtained with cultured cell lines. In addition, the possibility of isolating milligram quantities of specific glycoproteins from the tumors is examined.

EXPERIMENTAL

Materials. — Pronase CB was obtained from Calbiochem, La Jolla, CA 92037; controlled-pore glass from Electro-Nucleonics, Fairfield, NJ 07006; diethylaminoethyl (DEAE) cellulose and deoxyribonuclease (EC 3.1.4.5) from Sigma Chemical Co., St. Louis, MO 63178; ribonuclease (EC 2.7.7.16) from Boehringer Mannheim Biochemicals, Indianapolis, IN 46850; chondroitinase ABC (EC 4.2.2.4) from Miles Laboratories Inc., Elkhart, IN 46514; BioGel P-4 from BioRad Laboratories, Richmond, CA 94804; and lithium diiodosalicylate from Eastman Organic Chemical Div., Rochester, NY 14650. The tumors were obtained from C57B16/J mice that had been injected subcutaneously with B16 mouse melanoma cells grown in cultures.

Chromatography. — Exclusion chromatography was performed (a) on glyceryl, controlled-pore glass-beads CPG-240 (200–400 mesh) packed in columns (0.9 × 60 cm) with continuous vibration and eluted with 0.1M pyridinium acetate buffer (pH 5.2); (b) on BioGel P4 columns (0.9 × 54 cm) eluted with the same buffer; and (c) on Sephadex G-200 and Sepharose 4B columns (1.5 × 50 cm), and Sepharose 6B columns (1.5 × 85 cm) eluted with 50mM Tris–HCl buffer (pH 8.0). Ion-exchange chromatography was performed on (diethylamino)ethylcellulose (DE52) packed in a column (1.2 × 40 cm) and eluted with a linear gradient of 10mM to M pyridinium acetate buffer (pH 5.2) or in a column (0.9 × 10 cm) eluted stepwise with 0.5M, 0.8M, and M pyridinium acetate buffer (pH 5.2) (20 ml each). Wheat-germ agglutinin (WGA) was isolated and conjugated to Sepharose 4B as described earlier[4]; after loading of this sample, the WGA–Sepharose was first eluted with 50mM Tris–HCl buffer (pH 8.0), and then with Tris buffer containing 0.1M *N*-acetylglucosamine. Descending paper chromatography was performed in 2:3:1 (v/v) butanol–acetic acid–M NH_4OH. Methyl glycosides as their per-*O*-trimethylsilyl derivatives were separated by g.l.c. on 3.8% SE-30 as described by Clamp *et al.*[5], except that *N*-reacetylation was done according to Etchison and Holland[6].

GENERAL METHODS

Chondroitinase ABC digestions were done according to Saito *et al.*[7] Unsaturated disaccharides derived from chondroitin 4-sulfates were quantitatively determined by measuring their absorbance at 232 nm. Molecular weights of glycosaminoglycans were determined by equilibrium sedimentation[2,8]. Hydrolysis, prior to amino acid analysis, was carried out *in vacuo* with 6M HCl at 110° for 24 h. Protein was determined according to Lowry *et al.*[9], using crystalline bovine serum albumin as the standard. Total sialic acid was determined by the periodate–resorcinol method[10].

Cellulose acetate electrophoresis was carried out in the following electrolytes: LiCl or $ZnSO_4$[11], 0.2M butylamine[12], and calcium acetate[13]. Alcian Blue staining (0.1% in 0.5% acetic acid) was used for detecting glycosaminoglycans and sialoglycopeptides.

Reductive β-elimination was performed with M $NaBH_4$ in 0.1M NaOH for 72 h at 37°; the digest was neutralized with 4M acetic acid prior to analysis.

Extraction procedure. — The tumors (114 g, weight of wet material) were cleaned free of connective tissue, homogenized in acetone, and air-dried to yield a material (20.8 g) containing about 25% of protein and ~0.5% of sialic acid by weight. A portion (16.6 g) was extracted with 2:1 (v/v) chloroform–methanol, followed by ether. The resulting, defatted tumor powder (12.9 g) was sequentially digested with ribonuclease in 50mM Tris–HCl buffer (pH 7.2) for 50 h, with deoxyribonuclease in 50mM Tris–HCl buffer (pH 7.4) containing 10mM $MgCl_2$ for 47 h, and with Pronase (250 mg added in portions over a period of 7 days) in Tris–HCl buffer (pH 8.0) containing 10mM $MgCl_2$ and 10mM $CaCl_2$. These digestions were carried out at 37° in the presence of toluene. The digest was exhaustively dialyzed, and centrifuged to remove insoluble material, and the supernatant was lyophilized. The resultant product (392 mg; 14% of sialic acid) was dissolved in water (25 ml), and saturated cetylpyridinium chloride (CPC) added until no more precipitate was formed. The CPC precipitate was harvested by centrifugation, and sequentially extracted with sodium chloride (0.2M, 0.4M, 0.8M, and 2.0M) containing 0.1% of CPC. The salt extracts were freed of CPC and ionic components by dialysis at 37°, and lyophilized. The yields were 17.6 mg, 2.0 mg, 7.7 mg, and 4.3 mg, respectively.

The recovery of material in the CPC-precipitable fraction is comparable to that obtained with *in vitro* cultured cells which utilize D-[^{3}H]glucosamine as a metabolic precursor. The bulk of the nonprecipitable material consisted of glycopeptides, most of which appear to contain *N*-linked carbohydrate; they were not studied further. The CPC precipitate contained ~20% of the total sialic acid content, all of which being present in the 0.2M salt eluate.

A portion of the acetone powder (4.2 g) was defatted, treated with ribonuclease and deoxyribonuclease as just described, dialyzed, and extracted with lithium diiodosalicylate[14]; this extract was used for glycoprotein isolation.

RESULTS AND DISCUSSION

The 2.0M NaCl Fraction. — Examination of the 2.0M NaCl fraction (4.3 mg) by cellulose acetate electrophoresis in LiCl buffer gave the results shown in Fig. 1. The major band had the same mobility as chondroitin 4- or 6-sulfate, and the minor band had the same mobility as the mucin-type sialoglycopeptide of the 0.2M NaCl Fraction. Further electrophoretic studies employing $ZnSO_4$, calcium acetate, or butylamine as electrolytes confirmed the identity of the major band as either chondroitin 4- or 6-sulfate.

Examination by cellulose acetate electrophoresis in butylamine of the material,

after digestion with chondroitinase ABC, showed that the major band was completely digested. Descending paper chromatography of the digest in 2:3:1 (v/v) butanol–acetic acid–M NH_4OH showed the presence of the unsaturated disaccharide derived from chondroitin 4-sulfate; no other unsaturated disaccharide was present. These data identified the major component as chondroitin 4-sulfate.

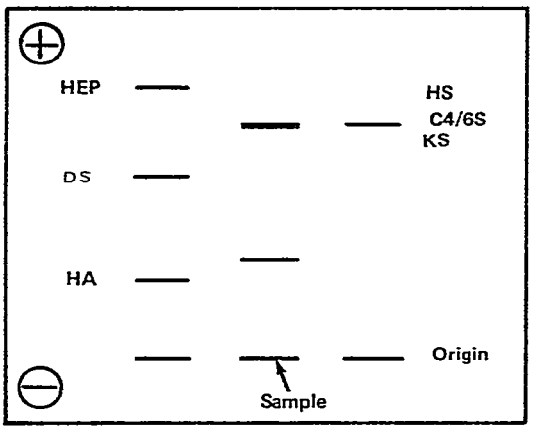

Fig. 1. Cellulose acetate electrophoresis of the 2.0M NaCl Fraction in 50mM LiCl–10mM HCl, pH 2.0 at 1 mA per cm for 20 min. The components were detected with the Alcian Blue stain. Abbreviations: Hep, heparin; HS, heparan sulfate; C4S, chondroitin 4-sulfate; C6S, chondroitin 6-sulfate; KS, keratan sulfate; DS, dermatan sulfate; and HA, hyaluronic acid.

The 2.0M NaCl eluate (2.5 mg) was chromatographed on a controlled-pore glass column, which had been calibrated with vitreous humor hyaluronic acid, cartilage chondroitin 4-sulfate, and D-glucuronic acid. Since analytical monitoring of the column effluent would have been wasteful considering the amount of material available, fractions were combined according to the profile of the standards as shown in Fig. 2, dialyzed, and lyophilized. Fractions 21–27 gave 0.73 mg, and Fractions 28–40 1.21 mg; this technique also removed the contaminating pigmented material that was eluted in Fractions 41–60. Examination of the fractions by cellulose acetate electrophoresis in $ZnSO_4$ showed that most of the bands that stained strongly with Alcian Blue were eluted in the high-molecular-weight region (Fractions 21–27) and that the area corresponding to a molecular-weight range of 13 000–20 000 (Fractions 28–40) contained most, if not all, of the weakly staining component. The identity of the high-molecular-weight fraction as chondroitin 4-sulfate was confirmed by cellulose acetate electrophoresis in $ZnSO_4$, and by paper chromatography of its chondroitinase ABC digest.

Analysis of the high-molecular-weight fraction by equilibrium sedimentation showed a mol. wt. range of $34\,670 \pm 2\,000$ to $69\,350 \pm 8\,000$. The results for amino acid analysis of the same fraction are shown in Table I. The major amino acids present were glycine, serine, glutamic acid, and aspartic acid. The first three amino

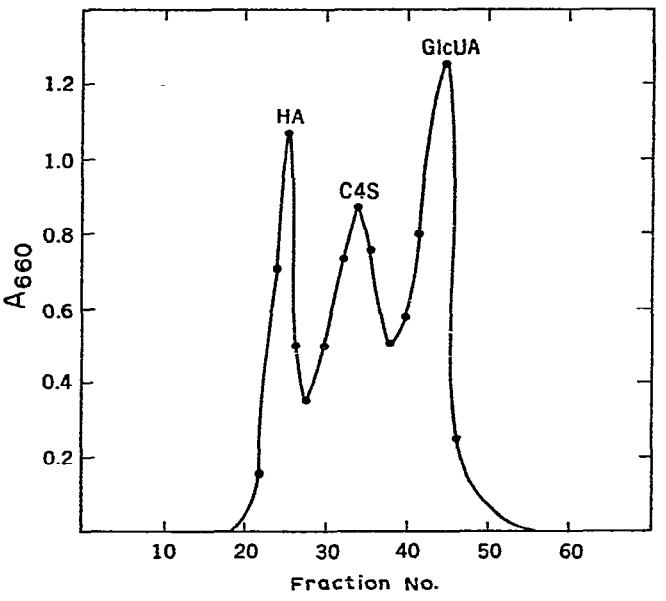

Fig. 2. Elution profile of vitreous humor hyaluronic acid, cartilage chondroitin 4-sulfate, and glucuronic acid (GlcUA) on a controlled-pore, glass-bead column (0.9 × 60 cm). The fractions were analyzed by the orcinol reaction for uronic acid. Abbreviations, see legend to Fig. 1.

TABLE I

AMINO ACID COMPOSITION OF THE MAJOR COMPONENTS OF 0.2M AND 2.0M NaCl FRACTIONS FROM MOUSE MELANOMA TUMORS

Amino acid	*(molar %)*	
	Peak 2[a] of 0.2M NaCl fraction	*Higher mol. wt. component of 2.0M fraction*
Lysine	1.3	n.d.[b]
Cysteic acid	0.7	0.6
Aspartic acid	6.8	4.6
Threonine	13.7	2.8
Serine	12.2	8.6
Glutamic acid	11.6	7.5
Proline	2.8	n.d.[b]
Glycine	15.0	11.2
Alanine	7.5	3.5
Valine	6.1	2.0
Isoleucine	1.3	1.2
Leucine	2.8	1.9
Tyrosine	0.7	n.d.[b]
Phenylalanine	1.3	0.8
Glucosamine	12.9	2.7
Galactosamine	3.4	52.7

[a]From WGA-column. [b]Abbreviation: n.d., not detected.

acids have been observed to be in the vicinity of the linkage between the carbohydrate chain and the protein core of cartilage proteoglycan[15]. From the results of the amino acid analysis, it can be seen that glucosamine is present in minor proportion relative to galactosamine.

Gas-liquid chromatographic analysis of the high-molecular-weight chondroitin 4-sulfate after methanolysis, *N*-reactylation, and silylation showed the presence of xylose, and possibly galactose. Xylose was identified by its retention times and by cochromatography with the trimethylsilyl derivatives of methyl xylosides. The sample gave a peak corresponding to one of the four peaks of a D-galactose standard, but quantitative determination was not performed. Cartilage chondroitin 4-sulfate, when examined under the same conditions, gave the same xyloside peaks together with one galactoside peak. The identification of galactose is only tentative, and it was not possible to deduce whether the structure Gal → Xyl → serine is present in this fraction.

The 0.8M NaCl Fraction. — Chromatography of the 0.8M NaCl Fraction (5.0 mg) on a controlled-pore glass column as just described gave fractions of high (0.61 mg), medium (2.39 mg), and low mol. wt. (1.55 mg). The high-molecular-weight fraction was examined by cellulose acetate electrophoresis in $ZnSO_4$ and showed one major band (>90%) and two minor bands. The major band had a mobility intermediate to that of heparan sulfate and chondroitin sulfates. An aliquot of this fraction was digested with chondroitinase ABC and re-examined by electrophoresis. The major band was completely digested leaving only the two minor bands. Examination of the digest by paper chromatography as described for the 2.0M NaCl Fraction showed the presence of unsaturated disaccharides corresponding to standards of unsaturated disaccharide 4-sulfate and disaccharides. Quantitative measurements gave a value of 1.0:1.4 for the ratio of sulfated to unsulfated disaccharides. Thus the high-molecular-weight component of the 0.8M NaCl Fraction is an undersulfated chondroitin 4-sulfate; its mobility on cellulose acetate electrophoresis is in accordance with this structure. Examination of this undersulfated chondroitin 4-sulfate by equilibrium sedimentation showed a mol. wt. range of $44\,000 \pm 2\,000$ to $88\,000 \pm 8\,000$. Gas-liquid chromatographic analysis of the high-molecular-weight fraction from the 0.8M NaCl Fraction indicated the presence of xylose and galactose. These sugars were identified by their retention times and cochromatography with standards. Other peaks of unknown identity were also obtained, presumably due to the impurities present as shown by the minor bands on cellulose acetate electrophoresis.

The 0.2M NaCl Fraction. — Cellulose acetate electrophoresis of the 0.2M NaCl Fraction in LiCl buffer gave two distinct bands and a third, diffuse band. After gel filtration on controlled-pore glass, examination of the fractions of different molecular weights by cellulose acetate electrophoresis showed that the diffuse band was eluted in the high-molecular-weight region. The intermediate-molecular-weight fraction gave two distinct bands in a ratio of 1:3 for the proportion of faster to slower moving bands, as shown in Fig. 3. Anion-exchange chromatography of this fraction on DEAE-cellulose and re-examination by cellulose acetate electrophoresis showed that

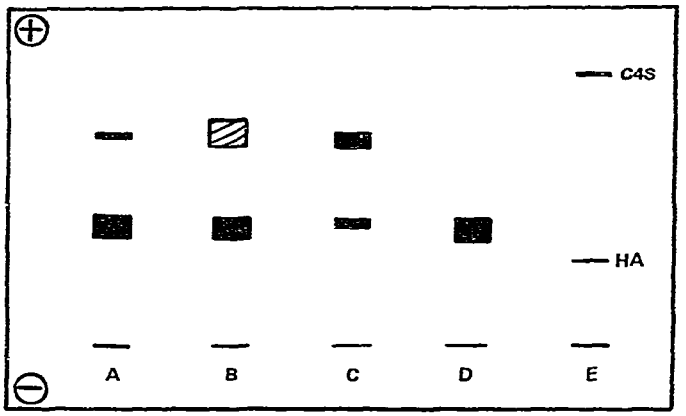

Fig. 3. Cellulose acetate electrophoresis of the intermediate-molecular-weight component of the 0.2M NaCl Fraction obtained by fractionation on a CPG column (Lane A). Conditions for electrophoresis were as described for the 2.0M fraction (see Fig. 1). Lane B, the fraction after treatment with chondroitinase ABC; Lane C, the portion not bound to the WGA–Sepharose; Lane D, the portion bound to WGA–Sepharose and eluted with GlcNAc; and Lane E, standard hyaluronic acid and chondroitin 4-sulfate. Abbreviations, see legend to Fig. 1.

the faster band was slightly more acidic than the slower band. Gas-liquid chromatographic analysis of the intermediate-molecular-weight fraction indicated the presence of mannose, galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and sialic acid, suggesting the presence of a mixture of sialoglycopeptides.

The sialoglycopeptides in the 0.2M NaCl Fraction were labeled on the sialic acid moiety by the periodate–tritiated borohydride technique[16]. Chromatography of the labeled sialoglycopeptides on BioGel P-4 before and after hydrolysis (50mM H_2SO_4, 80°, 1 h) proved that at least 95% of the label was on modified sialic acid residues (C-7 and -8 analogues). Chromatography on a WGA–Sepharose 4B column gave two fractions: one being eluted with Tris buffer alone (25%), and the other being eluted with the buffer containing *N*-acetylglucosamine (75%). This procedure was repeated on a preparative scale using the unlabeled material, and the fractions obtained were examined by cellulose acetate electrophoresis. The WGA-bound fraction gave only one band, whereas the unbound material contained all of the minor component and some of the major component, possibly due to overloading of the WGA–Sepharose column (Fig. 3). The two labeled fractions were examined on a BioGel P-4 column before and after alkaline borohydride treatment to determine the nature of the carbohydrate–peptide linkage (see Fig. 4). Before treatment, both samples were eluted in the void volume of this column. The WGA-bound fraction showed 81% of β-elimination, and the reduced oligosaccharides obtained had the same mobilities on a BioGel P-4 column as those derived from fetuin sialoglycopeptides treated similarly[17]. The material eluted in the void volume of the BioGel P-4 column did not bind to WGA. These results show that the WGA-bound sialoglycopeptide

contains mainly *O*-glycosyl linkages. By contrast, the unbound material gave 29% β-elimination, some of which may be derived from sialoglycopeptides that were not bound to WGA–Sepharose because of overloading. The fraction that did not bind to WGA–Sepharose was not further studied. The amino acid analysis of the WGA-bound fraction gave the data shown in Table I. As expected for an *O*-glycosyl sialoglycopeptide, serine and threonine were among the major amino acids present.

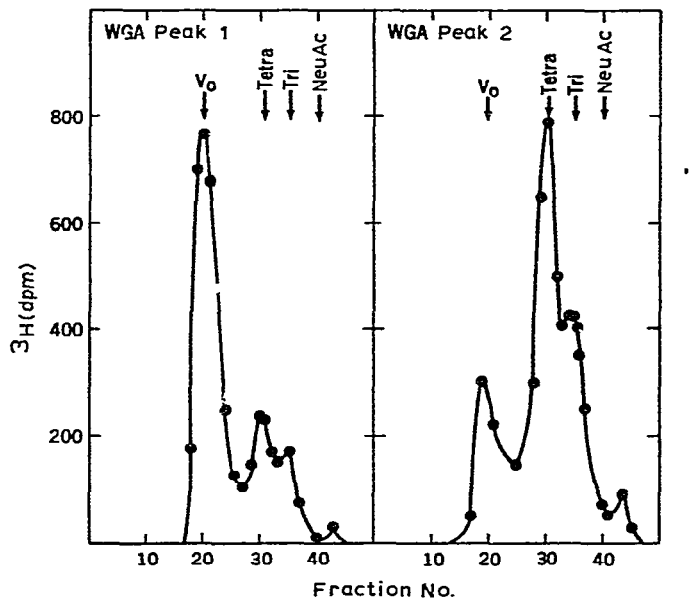

Fig. 4. Chromatography of the WGA–Sepharose subfractions WGA Peak I (material not retained on the WGA–Sepharose column) and WGA Peak II (material retained and eluted with 0.1M *N*-acetyl-glucosamine) of the 0.2M NaCl Fraction on BioGel P-4 column (0.9 × 54 cm), after treatment with alkaline borotritide. Aliquots of the fractions were analyzed for tritium activity. Peak positions of markers are indicated by arrows; Vo, Blue Dextran; Tetra and Tri, tetra- and tri-saccharides obtained by alkaline borotritide treatment of fetuin; NeuAc, *N*-acetylneuraminic acid.

The reduced oligosaccharides obtained from the 0.2M NaCl Fraction after β-elimination were purified by gel filtration on BioGel P-4 and examined by g.l.c.; sialic acid, galactose, and *N*-acetylgalactosaminitol were present in the ratio of 1.7:1.0:0.8. Thus, the major glycopeptide of the 0.2M NaCl Fraction is a sialoglycopeptide having *O*-glycosylically-bound oligosaccharides which consist of terminal sialic acid groups, and galactose and *N*-acetylgalactosamine (peptide-linked) residues; the carbohydrate chains are possibly 3 or 4 residues long. These characteristics are identical with those of the mucin-type sialoglycopeptide obtained from B16 melanoma cells grown in monolayer[3,4].

Isolation of the sialoglycoproteins. — In order to isolate the parent glycoprotein of this sialoglycopeptide, the tumors were extracted with lithium diiodosalicylate[14]. The water-soluble sialoglycoproteins were labeled in the sialic acid moiety by the

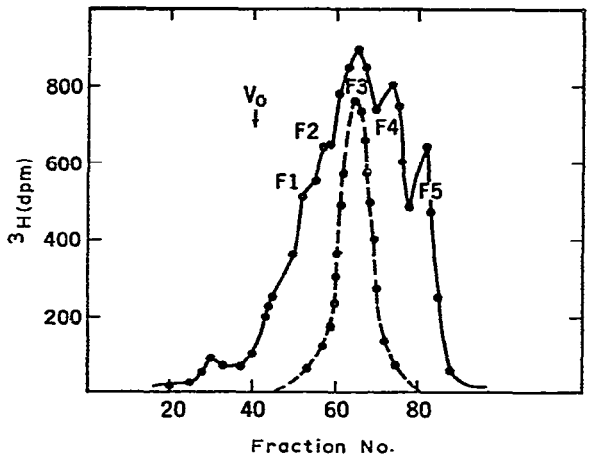

Fig. 5. Elution profiles of the sialoglycoproteins, prepared by solubilization of the tumor with lithium diiodosalicylate, on a Sepharose 4B column. The sialoglycoproteins were labeled with tritium by modification of the sialic acid groups. The solid line illustrates the profile of the whole fraction, and the broken line that of the fraction bound to a WGA–Sepharose column and eluted with GlcNAc.

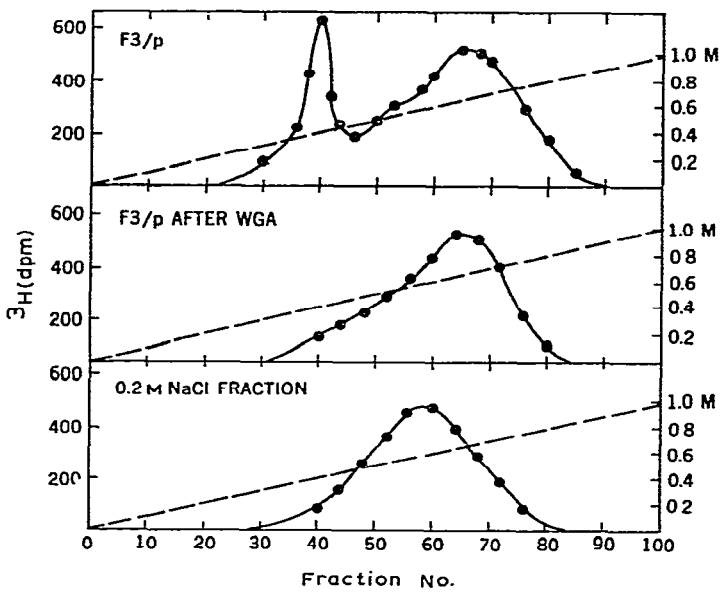

Fig. 6. Chromatography of ^{3}H-labeled sialoglycopeptides on a DEAE-cellulose column (1.2 × 40 cm). Elution was performed with a linear gradient of 10 mM to M pyridinium acetate, pH 5.2. Upper part: glycopeptides obtained by Pronase digestion of peak F3; middle part: the portion of the glycopeptides which bound to the WGA–Sepharose column (see Fig. 4); lower part: the 0.2 M NaCl Fraction obtained by CPC fractionation of the Pronase-digested tumor.

periodate–tritiated borohydride technique[16]. Chromatography of the labeled components on a Sepharose 4B column indicated the presence of several glycoproteins (see Fig. 5). After fractionation on WGA–Sepharose, the WGA-bound fraction gave only one peak (F3) when rechromatographed on Sepharose 4B. When F3 was rechromatographed on Sepharose 6B, DEAE-cellulose (DE52), or WGA–Sepharose, only one peak was obtained. Although Fraction F3 appeared homogeneous by these criteria, it must be remembered that these methods only monitor the presence of labeled sialoglycoprotein. The location of the label on sialic acid was identified by hydrolysis and chromatography on BioGel P-4.

Chromatography of Fraction F3, after Pronase digestion, on DEAE-cellulose showed a mixture of sialoglycopeptides (Fig. 6, upper part). These glycopeptides were chromatographed on WGA–Sepharose and the WGA-bound sialoglycopeptides, when examined on DEAE-cellulose, gave a single, broad peak (Fig. 6, middle part) comparable to that obtained when the 0.2M NaCl Fraction was examined on the same column (Fig. 6, lower part). When treated with alkaline borohydride, the WGA-bound sialoglycopeptides obtained from Fraction F3 gave results similar to those obtained from the major component of the 0.2M NaCl Fraction, 87% of β-elimination being observed in comparison to 81%. The amount of reduced trisaccharide obtained in the case of the 0.2M NaCl Fraction was higher, possibly because of the lengthier extraction procedure involved. Gel filtration on glass beads CPG-240 of the WGA-bound sialoglycopeptides from the 0.2M NaCl Fraction and from Fraction F3 gave identical profiles. Their behaviors on WGA–Sepharose, DEAE-cellulose, BioGel P-4, after alkaline–borohydride treatment, and controlled-pore glass all indicate that they are very similar. Thus, Fraction F3 appears to be the glycoprotein that gives rise to the sialoglycopeptide isolated in the 0.2M NaCl Fraction after Pronase digestion. It is interesting that, among the sialoglycoproteins of the B16 tumor, only one (F3) binds to WGA. Assuming identical incorporation of label per mole of sialoglycoprotein, it is calculated that Fraction F3 represents 40% (mole/mole) of the total sialoglycoproteins in the lithium diiodosalicylate extract.

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