# STUDIES ON GLYCOCONJUGATES IN HEALTH AND DISEASE

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A thesis presented for the degree of

Doctor of Science

University of Edinburgh

1983

CONTENTS

Unpublished Work

# A B S T R A C T

Glycoconjugates are widespread in nature and have been implicated in a variety of biological functions including energy source, protection, lubrication, structural support, cell adhesion, molecular and cellular recognition and receptors for hormones and viruses. In this thesis studies on the following glycoconjugates are presented; ovine submaxillary mucin, human respiratory mucins (normal and bronchial asthmatic), keratan sulfates (bovine cornea, human skeletal cartilage and chick allantoic fluid), human follicle-stimulating hormone and the glycoproteins and glycosaminoglycans of a variety of normal and cancer cells. The information obtained on the structures of these complex carbohydrate molecules is of value in understanding the biosynthesis, degradation, structure-function relationships and the changes in these macromolecules in disease. Studies on animal neuraminidases, an endoglycosidase and a lectin, all of which are important tools in the investigation of glycoconjugates, are also described.

The carbohydrate prosthetic group of ovine submaxillary mucin, consisting of N-acetylneuraminyl α2→6 N-acetylgalactosamine, was shown to be linked to serine and threonine and not to aspartic acid or glutamic acid as previously believed. The human respiratory mucins were shown to consist of a complex series of oligosaccharides composed of galactose, fucose, glucosamine, galactosamine and sialic acid. The high apparent molecular weight of the glycopeptides derived from the respiratory mucins could be due to clustering of the oligosaccharides on the peptide core. Our finding of the non-covalent association of lipids with the supposedly hydrophilic tracheobronchial mucins, suggests a role for the mucins in the clearing of lipids from the trachea. A new endoglycosidase capable of hydrolysing the O-glycosidic linkage between α-N-acetylgalactosamine and serine and threonine in mucins was isolated from Diplococcus pneumoniae culture filtrate and characterized. Among other uses, this enzyme became invaluable in the structural investigation of the mucin -type glycoproteins of cancer cells and in the elucidation of the antigenic determinant of epiglycanin.

The extensive studies on keratan sulfates led to the elucidation of several interesting and new structural features such as the sulfation of galactose, the presence of small amounts of mannose and the insolubility of the desulfated polysaccharide. Subsequently, this information was utilized by other researchers to understand the abnormal metabolism of keratan sulfate in diseases such as Marfan's syndrome and macular corneal dystrophy. Our discovery of the presence of glycosaminoglycans in animal cell nuclei was a surprise since these molecules have previously been considered secretory products found in the extracellular matrix. Several investigators have since confirmed the association of glycosaminoglycans with the nuclei. We have investigated the possibility that these molecules may also serve intracellular (for example, regulatory or modulatory) functions in addition to their accepted extracellular functions. Experiments designed to test the influence of anionic polysaccharides on endogenous DNA synthesis have yielded several provoking results. For example, concentrations of heparin, chondroitin sulfate H and heparan sulfate which stimulated DNA synthesis in normal cell nuclei inhibited DNA synthesis in cancer

cell nuclei. It was found that this differential effect was dependent on the molecular size, charge (degree of sulfation) and other structural features of the polysaccharides.

A new sensitive radioisotopic assay for neuraminidases was developed as part of our investigations on mammalian neuraminidases. A neuraminidase capable of acting on sialoglycoproteins but not gangliosides was detected in Golgi apparatus. The presence of this degradative enzyme in an organelle involved in biosynthesis suggests that it may be involved in processing of the newly synthesized glycoconjugates.

The wheat germ agglutinin, which was previously believed to be specific for N- acetylglucosaminyl residues was shown to be able to interact specifically with N-acetylneuraminyl and multiple N-acetylgalactosaminyl residues as well. On this basis we proposed that in the interaction between cell surface and wheat germ agglutinin, the non-reducing terminal sialic acid residues of glycoproteins and glycolipids play a more important role rather than the usually internal glucosamine residues. The new information has been very helpful in understanding the differential interaction of this lectin with normal and malignant cells and in the use of this lectin to select for non-tumorigenic and non-metastatic cell clones.

Over a period of ten years, extensive studies have been carried out to biochemically and immunologically characterize the glycosaminoglycans and glycoproteins derived from mouse melanoma, human melanoma, Morris hepatoma and appropriate control (non-malignant) cells. Chondroitin sulfate with properties distinct from those of the connective tissue counterpart was isolated from mouse melanoma cells. It was also observed that the heparan sulfates produced by cancer cells had a lower degree of sulfation compared to those produced by normal cells. A murine melanoma-associated antigen was purified and its glycoprotein structure established. A most important finding was the production of markedly increased quantities of mucin-type glycoproteins by a variety of tumorigenic cells compared to non-tumorigenic cells. The mucin-type glycoprotein from human melanoma cells was purified to homogeneity and partially characterized. A review by us on cell surface glycoprotein markers in neoplasia has been published. The research which is presently being carried out indicates that a new human cancer marker recently described (The Lancet, 3 July 1982, p.1.) is also a mucin-type glycoprotein.

Vol. 16, No. 4, 1964 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

# THE COMPLETE ENZYMIC DEGRADATION OF GLYCOPEPTIDES CONTAINING O-SERYL AND O-THREONYL LINKED CARBOHYDRATE

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Received May 18, 1964

In recent years a considerable amount of information on the molecular structure of glycoproteins has been published. Little is known, however, on the biosynthesis of these compounds and no information is available on the enzymic breakdown of glycoproteins to their individual constituents. In the present paper the purification and properties of a ß-N-acetylhexosaminidase and its action on glycopeptides containing O-seryl and O-threonyl linked N-acetylgalactosamine are described.

## EXPERIMENTAL AND RESULTS

### Purification and Properties of ß-N-Acetylhexosaminidase

The purification of the enzyme present in bovine spleen homogenates was carried out by fractional precipitation with ammonium sulfate (crude enzyme) followed by chromatography on TEAE and CM Cellulose columns (highly purified enzyme). The anomeric specificity of the enzyme was shown to be of the ß-type. Several natural and synthetic glycosides were found to be substrates for the enzyme (see also Weissmann et al., 1964). The $K_m$ values x $10^3$ for ß-phenyl-N-acetyl-D-glucosaminide, ß-phenyl-N-acetyl-D-galactosaminide and ß-p-nitrophenyl-N-acetyl-D-glucosaminide were 2.0, 1.1 and 1.4 respectively. The enzyme requires for its activity a terminal non-reducing

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N-acetylhexosamine residue. The increase in specific activity, referred to the homogenate as unity, was 42 fold for the crude enzyme and 20 000 fold for the highly purified enzyme.

Preparation and Properties of Glycopeptides from OSM

Ovine submaxillary gland glycoprotein (OSM) was prepared as previously described (Graham and Gottschalk, 1960) with the modification that the precipitation of glycoproteins and proteins to be discarded was carried out at pH 4.5 instead of pH 4.0. Yield 5% of the dry weight of the glands. The compound displayed a single and almost symmetrical peak in the analytical centrifuge. It contained about 40% carbohydrate (as residues) consisting of equimolar amounts of N-acetylneuraminic acid and N-acetylgalactosamine. 250 mg OSM was exhaustively treated with Pronase and the digest fractionated on a Sephadex G25 column. The pooled fractions of the sialoglycopeptide peak reacting with both ninhydrin and resorcinol were submitted to neuraminidase action and the enzyme was removed by a second passage through a Sephadex G25 column.

The sialic acid-free glycopeptides were separated from sialic acid on a Dowex I-X2 (formate form) column and the effluent was concentrated to dryness. Yield about 50 mg containing 50% of the galactosamine of OSM. On the Dowex I column about 35% of the applied glycopeptides, expressed in terms of galactosamine, were retained. The quantitative analysis of the glycopeptide fraction is shown in Table I. The near lack of dicarboxylic acids in the glycopeptide fraction (OSM contains 0.84 moles dicarboxylic acids/mole galactosamine) would indicate that a considerable amount of the glycopeptides, applied to the Dowex I column, contained aspartyl and glutamyl residues with unsubstituted ß- and $\gamma$-carboxyl groups respectively and were therefore retained on the column. In fact, when the column was eluted with 2 N acetic acid, analysis of the eluate showed the presence of 20% of the total dicarboxylic acids. Another 58% of dicarboxylic acids was found in the pool of free amino acids and small peptides emerging from the first Sephadex column

Table I. Amino acid composition of glycopeptide fraction
(expressed in moles per 1 mole of galactosamine)

| | | | |
|---|---|---|---|
| Aspartic acid | 0.03 | Proline | 0.36 |
| Glutamic acid | 0.08 | Glycine | 0.56 |
| Serine | 0.62 | Alanine | 0.30 |
| Threonine | 0.67 | Valine | 0.17 |
| Lysine | 0.01 | Leucine | 0.07 |
| Arginine | 0.09 | | |

after the sialoglycopeptide peak. The average molecular weight of the glycopeptides was about 660 (potentiometric titration) and the molar ratio amino acids/hexosamine (after hydrolysis of the glycopeptides) was found to be 3.3. These data would indicate that on an average the glycopeptides were tetrapeptides with 1.3 residues of N-acetylgalactosamine attached.

Type of Linkage between N-Acetylgalactosamine and Peptide

It is evident from Table I that in the glycopeptides only serine and threonine are conceivable as sugar acceptors. On treatment of the glycopeptide fraction with 0.5 N NaOH at $0^{\circ}$ for 20 hr (see Anderson et al., 1963) followed by acid hydrolysis, only 504 μmoles of serine and 576 μmoles of threonine were recovered from 900 and 960 μmoles of serine and threonine respectively present in 1 gram of untreated glycopeptide, whereas the other amino acids were recovered quantitatively. When the experiment was extended to 68 hr, 456 and 475 μmoles of serine and threonine respectively were recovered. The combined loss of 929 μmoles of serine and threonine coincided with the appearance of 900 μmoles of free N-acetylhexosamine in the assay prior to acid hydrolysis. When DL-seryl-glycyl-glycinamide and L-seryl-leucyl-leucine were treated similarly, the losses of serine did not exceed 10%. These data can only be interpreted as resulting from ß-elimination of N-acetylhexosamine residues linked O-glycosidically to the hydroxyl groups of peptide-bonded serine and threonine residues.

Enzymic Degradation of Glycopeptides

When the glycopeptides were treated with crude enzyme added in portions (0.025 M citrate buffer, pH 4.3, 37°), 40.0, 75.9 and 94.7% of the total hexosamine were released after 48, 96 and 144 hr respectively; paper chromatography of the digest revealed in addition to free N-acetylgalactosamine all the amino acids listed in Table I. The presence of peptides in the digest freed from proteins was seen from a 40% increase of the ninhydrin value (corrected for hexosamine) after acid hydrolysis of the digest. The highly purified enzyme released under same conditions 6.7, 9.8 and 13.2% respectively of the total hexosamine. From sialoglycopeptides only 4.5 and 6.5% of hexosamine were split off after 54 and 97 hr incubation respectively with the crude enzyme.

## DISCUSSION

These results indicate that in OSM, as prepared above, at least 50% of the total hexosamine is linked ß-O-glycosidically to the OH-groups of seryl and threonyl residues of the peptide and that this linkage is susceptible to N-acetylhexosaminidase. The enzymic cleavage takes place only after removal of sialic acid and after shortening of the glycotetrapeptide by at least one or two amino acid residues. The shortening is effected by peptidases the presence of which in the crude enzyme was also evidenced by its action at pH 4.3 on L-seryl-L-leucyl-L-leucine. This type of peptidases is quite distinct from the known exopeptidases. The occurrence of these peptidases and ß-N-acetylhexosaminidase in the same tissue and their activity at the same pH favours the view that the sequence of reactions shown in the diagram may be the physiological degradation mechanism.

DIAGRAM

Sialoglycopeptides

↓ NEURAMINIDASE

GalNAc-Peptides + N-acetylneuraminic acid
(Glycopeptides)

↓ PEPTIDASES

O-Seryl-N-Acetylgalactosaminide + amino acids

↓ ß-N-ACETYLGALACTOSAMINIDASE

Serine + N-acetylgalactosamine

A similar scheme would hold for O-threonyl-N-acetyl-galactosaminide.

The relative proportion of glycosidic-ester linkages (Graham et al., 1963) and O-seryl (threonyl)-glycosidic linkages in various OSM preparations is at present under investigation.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. Peter Anders, M-P-I für Virusforschung, for the quantitative amino acid analyses. This research has been supported in Part by Grants GM 10917-02 and AI 04954-02 of the U. S. Public Health Service and by the Deutsche Forschungsgemeinschaft.

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Reprinted from
*Biochimica et Biophysica Acta*
Elsevier Publishing Company
Amsterdam
Printed in The Netherlands



BBA 85041

STUDIES ON GLYCOPROTEINS

# XI. THE O-GLYCOSIDIC LINKAGE OF *N*-ACETYLGALACTOSAMINE TO SERYL AND THREONYL RESIDUES IN OVINE SUBMAXILLARY GLAND GLYCOPROTEIN

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(Received November 5th, 1964)

SUMMARY

Ovine submaxillary glycoprotein (OSM) was digested exhaustively with pronase and the sialoglycopeptides separated by gel filtration. By subsequent neuraminidase (EC 3.2.1.18) treatment and further purification, glycopeptides of average molecular weight 660 and containing 50% of the total galactosamine of OSM were obtained. Serine and threonine were the only amino acids with a functional group in the side chain present in a concentration high enough to accommodate all hexosamine residues. Treatment of the glycopeptides with 0.5 N NaOH at 0° or 22° resulted in the release of *N*-acetylgalactosamine coincident with the loss of an equimolecular amount of hydroxyamino acids. Most likely the carbohydrate was released by the mechanism of $\beta$-carbonyl elimination. Some evidence for the formation of an unsaturated compound, presumably $\alpha$-aminoacrylic acid, was afforded spectrophotometrically.

It is concluded that in OSM at least 50% of the prosthetic groups are joined glycosidically to the hydroxyl groups of serine and threonine.

When native OSM was treated at pH 8.0 and 42° for 120 h, about one-third of the prosthetic groups was released. After this treatment the isolated, non-dialyzable residual glycoprotein reacted strongly positive in the Warren test. The mechanism of the reaction is discussed.

## INTRODUCTION

Some evidence suggesting the presence in glycoproteins of animal origin of O-glycosidic linkages involving the hydroxyl groups of serine and threonine residues was presented in 1962 from the laboratories of Blix, Gottschalk and Pigman. In the former laboratories OSM was used for the investigation. This glycoprotein contains

Abbreviations: OSM, ovine submaxillary gland glycoprotein; NANA, *N*-acetylneuraminic acid.

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several hundred prosthetic groups of the structure *N*-acetylneuraminyl (2 → 6) *N*-acetylgalactosamine[1]. MURPHY[2] submitted OSM, pretreated with 0.01 N NaOH at 80° for 4 h, to hydrazinolysis. The resulting mixture of glycopeptide hydrazides was fractionated by high-voltage paper-electrophoresis and further by ion-exchange column chromatography. By this procedure a glycopeptide, containing 2% (w/w) of the galactosamine of native OSM, was isolated; it yielded on acid hydrolysis glycine, serine, alanine, valine and galactosamine in the molar ratios 1:1:3:1:1. This result strongly suggested that in the isolated glycopeptide galactosamine was attached to serine, most likely through an O-glycosidic linkage. BLIX[3] obtained by papain (EC 3.4.4.10) digestion of OSM and fractionation of the resulting dialysable glycopeptides a fraction (0.25% w/w of native OSM) which on analysis showed the following molar composition: — proline (8), threonine (5), alanine (5), glutamic acid (3), glycine (3), serine (1), *N*-acetylgalactosamine (6). Similar results were reported for glycopeptides prepared from bovine submaxillary glycoprotein by HASHIMOTO *et al.*[25].

Shortly afterwards, conclusive proof for the presence in ovine and bovine submaxillary gland glycoproteins of O-glycosidic linkages involving the hydroxyl groups of seryl and threonyl residues was presented in short communications independently by three laboratories. The workers concerned made use of the ease with which on mild alkali treatment an alkoxide (sugar residue) is released from the O-substituted, peptide-bonded α-amino-β-hydroxycarboxylic acid residues by the mechanism of β-carbonyl elimination, first demonstrated on chondromucoprotein from bovine nasal septa by ANDERSON *et al.*[4]. BHAVANANDAN *et al.*[5] treated OSM exhaustively with pronase and fractionated the digest by gel filtration. The sialoglycopeptide fraction was submitted to neuraminidase (EC 3.2.1.18) action and the resulting glycopeptides were separated from the enzyme and free sialic acid by gel filtration and anion-exchange resins respectively. On mild alkali treatment at 0° of the short glycopeptides which contained only minute amounts of dicarboxylic acids, *N*-acetylhexosamine was liberated concomitantly with the disappearance of an equimolar amount of α-amino-β-hydroxycarboxylic acids. TANAKA *et al.*[6] treated bovine submaxillary glycoprotein at 5° with mild alkali in the presence of sodium borohydride. The result was a progressive release of carbohydrate, a decrease in serine and threonine, an increase in alanine (corresponding to the decrease in serine) and the appearance of a small amount of α-aminobutyric acid. The decrease of bound hexosamine proceeded initially at a higher rate than the decrease of the α-amino-β-hydroxycarboxylic acids. Similar results for bovine submaxillary mucin were reported by ANDERSON *et al.*[7]. Finally HARBON *et al.*[8] observed as a result of OSM treatment at pH 12.8 and 70° a decrease of bound hexosamine by 83% of the total and a loss of serine and threonine. Since under these conditions the serine and threonine content of lysozyme (EC 3.2.1.17) which is free of sugar also diminished by about 15%, the quantitative relationship between sugar released and α-amino-β-hydroxycarboxylic acids disappeared could not be elucidated. The formation of an unsaturated serine derivative (α-aminoacrylic acid) in the process was shown by the addition of sulphite to the alkali-treated OSM. 54% of the missing serine was transformed by this addition to cysteic acid.

In the present paper a detailed account will be given of the preparation, composition and average molecular weight of glycopeptides from OSM and of the effect of mild alkali treatment on these glycopeptides.

## EXPERIMENTAL

### *Materials*

OSM was prepared as previously described[9] for bovine submaxillary glycoprotein with the modification that the precipitation of glycoproteins and proteins to be discarded was carried out at pH 4.5 instead of pH 4.0. The successive aqueous extracts of the glands were processed separately. Total yield 5% of the dry weight of the glands. On sedimenting the OSM preparation, dissolved in 0.1 M phosphate buffer (pH 7.6), in a Spinco Model E analytical ultracentrifuge, using a double sector cell, a single peak with no appreciable asymmetry was observed.

The pronase preparation used was a product of Sigma, St. Louis (protease from *Streptomyces griseus*, repurified, Type VI).

Neuraminidase, purified, Type V from *Clostridium perfringens* was also a product of Sigma, St. Louis.

Sephadex G-25 was the preparation from Pharmacia, Uppsala, Sweden.

Resins used were Dowex 50-X4 (50–100 mesh), Dowex 1-X2 (50–100 mesh) and Amberlite IR-4B (50–100 mesh).

Crystalline methoxyneuraminic acid was a gift from Professor H. FAILLARD, Cologne.

### *Methods*

Measurements of pH were made with a Radiometer type TTT1b equipped with a glass electrode type G 222C.

Bound and total (bound plus free) NANA were determined by the resorcinol method of SVENNERHOLM[10] using crystalline NANA as standard. Free NANA was estimated by the thiobarbituric acid method[11].

*N*-Acetylhexosamine was determined according to Morgan and Elson as modified by REISSIG *et al.*[12].

Hexosamine was determined as described previously[13]. Prior to assay the material was hydrolysed with 3 N HCl at 100° for 8 h in a sealed tube. The hydrolysate was dried *in vacuo*. On the basis of model experiments a correction for the destruction of 10% galactosamine was applied.

The non-amino sugars were determined according to GOTTSCHALK AND ADA[14].

The ninhydrin value of the untreated glycopeptides was determined according to MOORE AND STEIN[15] with L-leucine as standard and the result was expressed as μmoles of leucine. The total amino acid content of the glycopeptides, after hydrolysis with 6 N HCl for 24 h at 110° in an atmosphere of nitrogen in sealed tubes, was determined with the ninhydrin reagent of MOORE AND STEIN; L-leucine was the standard. The experimental figure was corrected for the proline content (see Table II) and for the contribution made to the ninhydrin value by the degradation products of *N*-acetylgalactosamine. It was found that galactosamine–HCl, when treated with 6 N HCl at 110° for 16 h, gave in the ninhydrin test a molar absorbancy equivalent to that of the leucine standard. The quantitative analysis of the individual amino acids in the glycopeptide hydrolysate was carried out with an amino acid analyser Spinco Model 120.

Gel filtration: Sephadex G-25 was allowed to swell in 0.05 M NaCl and the fines were removed by repeated decantation. The suspension was slurried into the

column (83 cm × 2.1 cm). The column was washed first with 0.1 M acetic acid until free of chloride and then with degassed distilled water until neutral. Between runs the column was washed with 0.1 M acetic acid followed by water. Monoamino-dicarboxylic acids were determined as described previously[16].

Paper chromatography: Whatman No. 1 paper was used with the solvents (a) *n*-butanol–pyridine–water (6:4:3, v/v), (b) *n*-propanol–pyridine–water (6:4:3, v/v), (c) *n*-butanol–acetic acid–water (12:3:5, v/v), (d) phenol–water–ammonia, prepared by adding 40 ml of 0.1% ammonia to 160 g of phenol. The spray reagents were the same as previously described[13]. To locate peptides, the spray of PAN AND DUTCHER[17] was used.

For potentiometric titration the micrometer syringe "Agla" (total volume 500 $\mu$l), Burroughs Wellcome & Co., London, and the above mentioned Radiometer were used.

High-voltage paper electrophoresis was performed in pyridine–acetic acid–water buffer (1:10:289, v/v) at pH 3.6 and 4° for about 90 min, 80 V/cm, with the apparatus designed by ZILLIG *et al.*[18] and manufactured by A. Hölzel, Munich 2, Blutenburgstr. 96.

*Preparation of glycopeptides*

*First step: Pronase digestion of OSM.* 250 mg of OSM was dissolved in 50 ml of 0.01 M $CaCl_2$, saturated with toluene, and the pH was adjusted to 8.0 with 1 N NaOH. The mixture was digested at 42° with 14 mg pronase, added in three portions at 24 h intervals. The incubation lasted for a total of 120 h; the pH was maintained at 8.0. The progress of the digestion was followed by the quantitative ninhydrin reaction. The digest, after neutralization, was freed from insoluble material by filtration. NANA was retained on the molecule because it facilitated fractionation of the digest by increasing the molecular size of the glycopeptides and by affording a sensitive and easily detectable marker of the sialoglycopeptide fraction.

*Second step: Fractionation of digest.* 25 ml of the above digest was concentrated in a rotary evaporator at 27° to about 2 ml volume. The concentrate was applied to the Sephadex G-25 column which was developed with air-free distilled water. The flow-rate was adjusted to about 15 ml/h and 2.5 ml fractions were collected automatically. The elution pattern is shown in Fig. 1. When Fractions 1–37 (pool A) were screened for protein with the Folin reagent, they gave a negative test; even after concentration to a small volume the test was only faintly positive (see RESULTS). The Fractions 38–64 reacting with both resorcinol and ninhydrin were pooled and concentrated to about 10 ml volume (pool B). The fractions from 65 onwards, reacting only with ninhydrin, were pooled separately (pool C). The remaining half of the digest was fractionated similarly and the resulting pools were combined with the corresponding pools from the first half of the digest.

*Third step: Treatment with neuraminidase.* To the 20 ml of sialoglycopeptides (pool B) were added 0.25 ml of 1 M $CaCl_2$ and water to a final volume of 25 ml and the pH was adjusted to 5.7. Two additions of 2.5 mg neuraminidase each were made at times 0 and 24 h and the mixture was kept in the presence of toluene at 37° for 48 h. At the end of this period there was no appreciable difference between free and total *N*-acetylneuraminic acid indicating quantitative release of the latter.

*Fourth step: Separation of glycopeptides.* The glycopeptides resulting from step 3

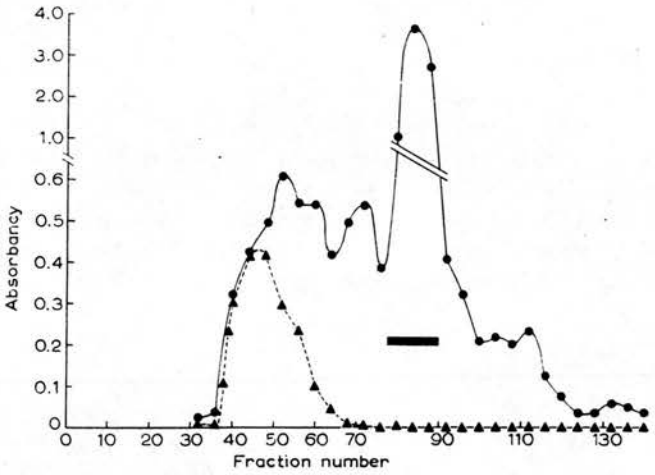



Fig. 1. Elution pattern from a Sephadex G-25 column (83 × 2.1 cm) of the pronase digest of OSM (corresponding to 94 mg OSM). Elution carried out with air-free distilled water. Fractions of 2.5 ml were collected. ●—●, absorbancy at 570 mμ in the MOORE AND STEIN[15] test (0.1 ml aliquot in final volume of 3.5 ml); ▲— — —▲, absorbancy at 580 mμ in the resorcinol test (0.1 ml aliquot in final volume of 2.5 ml). The bar denotes fractions containing chloride.

were separated from neuraminidase and salt by another passage through Sephadex G-25 as above. The fractions containing glycopeptides (ninhydrin positive) emerged from the column together with free sialic acid. To remove sialic acid the mixture, concentrated to 15 ml volume, was applied to a Dowex 1 (formate) column (12 cm × 1.1 cm) and the effluent was recycled once. The column was washed with 85 ml water and the pooled effluent and washings were concentrated to a small volume and finally dried in a desiccator at 0°. Yield about 50 mg. The recovery from the individual steps is shown in Table I.

TABLE I

RECOVERY OF HEXOSAMINE BOUND TO PEPTIDE FROM THE INDIVIDUAL STEPS OF THE PREPARATION OF GLYCOPEPTIDES FROM 250 mg OSM

The figures of this table refer to preparation No. 15.

| *Material* | *Treatment* | *Product* | *Hexosamine (μmoles)* | *Recovery (%)* |
|---|---|---|---|---|
| OSM | None | OSM | 187* | 100 |
| OSM | Pronase; Fractionation on Sephadex G-25 | Sialoglyco-peptides | 152 | 81.3** |
| Sialoglycopeptides | Neuraminidase; Fractionation on Sephadex G-25 | Glycopeptides and free NANA | 147 | 78.6 |
| Glycopeptides and free NANA | Dowex-1 | Glycopeptides | 92 | 49.2 |

* This figure is not corrected for the moisture content of OSM.
** At this stage the recovery of NANA was nearly quantitative.

RESULTS

*Carbohydrate analysis of OSM*

The composition of the carbohydrate of OSM, prepared as above, was similar to the Australian OSM. It contained 24.2% sialic acid (expressed as NANA) and 14.6% hexosamine (expressed as free galactosamine base); both values are corrected for moisture content of OSM. Practically all sialic acid was NANA and practically all hexosamine was galactosamine, as seen on paper chromatograms. There were also present 0.2% galactose, 0.2% mannose, 0.25% fucose and traces of glucosamine.

*Characterization of glycopeptides*

*Quantitative analysis*

The quantitative analyses of two representative glycopeptide preparations are given in Table II.

TABLE II

COMPOSITION OF GLYCOPEPTIDE FRACTION FROM ENZYMATIC DIGEST OF OSM

Figures expressed in moles per 1 mole of hexosamine.

| *Amino acid* | *Preparation* | | *Amino acid* | *Preparation* | |
|---|---|---|---|---|---|
| | *15* | *17* | | *15* | *17* |
| Aspartic acid | 0.03 | 0.05 | Proline | 0.36 | 0.40 |
| Glutamic acid | 0.08 | 0.13 | Glycine | 0.56 | 0.70 |
| Serine | 0.62 | 0.66 | Alanine | 0.30 | 0.40 |
| Threonine | 0.67 | 0.62 | Valine | 0.17 | 0.21 |
| Lysine | 0.01 | trace | Leucine | 0.07 | 0.10 |
| Arginine | 0.09 | trace | | | |

*Molecular weight*

About 10 mg of the glycopeptide preparation was dissolved in 5 ml of 0.1 M KCl. This solution was titrated potentiometrically with 0.1 N NaOH at 20°. The mixture was stirred magnetically and a continuous stream of $N_2$ was passed over the surface. In the critical region between pH 5.0 and 10.0 the alkali was added in 20 $\mu$l portions between the individual pH measurements. Appropriate corrections were made for the alkali consumption of the 0.1 M KCl blank. From the plot of alkali consumption against pH it was seen that between pH 5.0 and 9.75 the mixture of glycopeptides contained essentially one type of titratable group ($-NH_3^+$). The plot for the back titration using 0.1 N HCl almost coincided with the curve obtained with 0.1 N NaOH. From the alkali consumed within this range the molecular weights were calculated (see Table III). Each figure represents the average molecular weight of the respective glycopeptide preparation.

*Ninhydrin values before and after hydrolysis*

To characterize further the glycopeptides the ninhydrin value before and after hydrolysis (see METHODS) and the hexosamine content after hydrolysis were

determined. From the figures in Table III it would appear that the average glycopeptide, as prepared in our laboratory, is a tetrapeptide with 1.3 residues *N*-acetylgalactosamine attached to it.

TABLE III

MOLAR RATIOS NINHYDRIN VALUE*/HEXOSAMINE IN PREPARATIONS OF GLYCOPEPTIDES FROM OSM AND MOLECULAR WEIGHTS

| | *Molar ratios* | | |
|---|---|---|---|
| *Preparation* | *Before hydrolysis* | *After hydrolysis* | *Mol. wt.* |
| No. 7 | 0.48 | 3.29 | |
| No. 11 | 0.63 | 3.03 | |
| No. 14 | 0.65 | 3.19 | 644 |
| No. 15** | 0.52 | 3.41 | 677 |
| No. 17 | 0.51 | 3.39 | 658 |
| No. 18 | 0.57 | 3.49 | |

* Value expressed as leucine.
** Pool of three preparations.

When glycopeptide preparation No. 14 was treated with pronase at pH 8.0 and 42° for 88 h, the ratios before and after hydrolysis changed to 0.75 and 2.55, respectively, indicating a further shortening of the glycopeptides. It may be mentioned that this shortening of the peptide was possible only because the glycopeptide was free from sialic acid (see third step of glycopeptide preparation). This was shown by the following experiment. Sialoglycopeptides, obtained from fractionation (on Sephadex column) of the pronase digest of OSM, were submitted to two further pronase treatments and fractionations. After removal of the bound sialic acid, the ratios of the final glycopeptide (Table III, preparation No. 18) did not reveal any difference from those prepared by the standard procedure.

*High-voltage paper electrophoresis*

When electrophoretograms of the glycopeptides were sprayed with the peptide reagent and the periodate–benzidine reagent, a series of spots was seen which had migrated towards the cathode and which reacted with both reagents.

*Paper chromatography*

Paper chromatograms of the glycopeptides in solvents a, c and d (see METHODS) did not reveal free amino acids or free *N*-acetylgalactosamine. Paper chromatograms of the acid hydrolysate of the glycopeptides (6 N HCl, 110°, 24 h) in solvents a and c showed the amino acids listed in Table II. The relative intensities of the spots roughly agreed with the molar ratios in Table II.

*Balance of monoaminodicarboxylic acids*

250 mg of OSM contained 156.2 μmoles of monoaminodicarboxylic acids: this figure is uncorrected for the 11% moisture of the lyophilized OSM. When pool C (see METHODS) was hydrolysed with 6 N HCl at 110° for 24 h and analysed for

dicarboxylic amino acids, 91.0 μmoles were recovered. When the Dowex-1 column, used in the fourth step of glycopeptide preparation, was eluted with 2 N acetic acid, 29.9 μmoles of dicarboxylic amino acids were found in the acid hydrolysate of the eluate. In the glycopeptide fraction (Table II) 10.1 μmoles of aspartic and glutamic acids were present. The total recovery of monoaminodicarboxylic acids, referred to 250 mg OSM, was therefore 83.9%. The above figures refer to preparation 15.

*Pool C*

The major components of pool C are free amino acids. A minor contribution is made by small peptides, as indicated by an increase of the ninhydrin value of the pool after acid hydrolysis by not more than 26%. Tests for hexosamine after acid hydrolysis of the pool were always negative.

*Self-digestion of pronase*

The observation that pool A of the fractionated OSM–pronase digest (second step of glycopeptide preparation) did not reveal appreciable amounts of protein prompted an investigation into the self-digestion of pronase.

6.83 mg of pronase, dissolved in 25 ml of 0.01 M $CaCl_2$ and the pH adjusted to 8.0, was incubated for 3 days at 42° in the presence of toluene. After neutralization and removal of some insoluble material by filtration, the concentrated digest was fractionated on a Sephadex G-25 column under the conditions described in the second step of glycopeptide preparation (see METHODS). The elution pattern is shown in Fig. 2. In order to assess the size of polypeptides and/or peptides emerging from

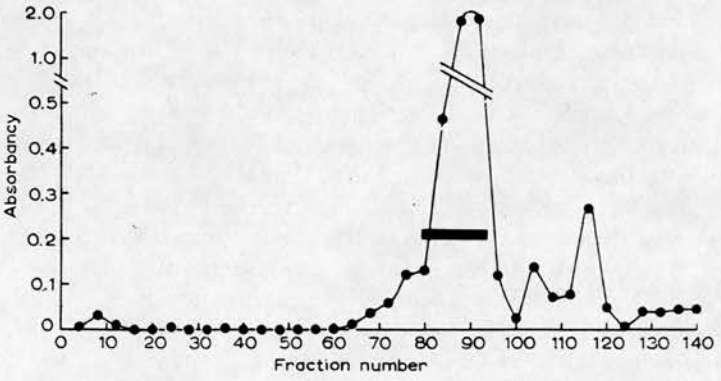



Fig. 2. Elution pattern from a Sephadex G-25 column (83 × 2.1 cm) of the products of self-digestion of 6.83 mg of pronase incubated at 42° for 3 days in 25 ml of 0.01 M $CaCl_2$ at pH 8. Elution carried out with air-free distilled water. Fractions of 2.5 ml were collected. ●—● absorbancy at 570 mμ in the MOORE AND STEIN[15] test (0.5 ml aliquot in final volume of 3.5 ml). The bar denotes fractions containing chloride.

the column and to obtain information on their distribution over the fractions corresponding to pools A, B and C (see *Second step* of *preparation of glycopeptides* under METHODS), the pools a, b, and c (see Table IV) were analysed for ninhydrin value before and after hydrolysis with 6 N HCl at 110° for 24 h. It would appear from

Table IV that no appreciable amounts of protein or larger polypeptides are present in pool a. The pools a, b and c consist of short peptides decreasing in size from pool a to pool c. Pool c is essentially a mixture of free amino acids and small peptides and is in that respect similar to pool C from the OSM–pronase digest. In pool c the

TABLE IV

ANALYSIS OF THE POOLS OBTAINED FROM FRACTIONATION OF THE PRONASE SELF-DIGEST

| *Pool* | *Fractions* | *Total ninhydrin value (expressed in μmoles leucine)* | | *Ninhydrin value (after hydrolysis) expressed in % of total ninhydrin value of a + b + c* |
|---|---|---|---|---|
| | | *Before hydrolysis* | *After hydrolysis* | |
| a | 1– 37 | 0.40 | 1.62 | 5.2 |
| b | 38– 64 | 0.34 | 1.25 | 4.0 |
| c | 65–145 | 16.30 | 28.10 | 90.8 |

increase in ninhydrin value after acid hydrolysis amounted to about 72%. It can be calculated from Table IV that contamination of the sialoglycopeptides, resulting from pronase digestion of OSM and collected in pool B, by products of pronase self-digestion (pool b) is negligible. The contribution of pool b (after hydrolysis) from the pronase autodigest to the total ninhydrin value of pool B (after hydrolysis) was less than 1%.

*Alkali treatment of glycopeptide fraction*

The analysis of the glycopeptide fraction (see Table II) suggested the hydroxyl groups of serine and threonine as acceptors for the *N*-acetylgalactosamine residues. It is well known[19] that glycosidic linkages with a carbonyl group in $\beta$-position are alkali-sensitive.

On treatment of glycopeptide preparation 17 with 0.5 N NaOH at 0° 52.0, 65.7 and 69.2% *N*-acetylhexosamine were released after 48, 96 and 120 h, respectively. When carrying out the experiment at room temperature (about 22°), 66.6 and 79.6% *N*-acetylhexosamine were liberated after 24 and 48 h, respectively. These figures are corrected for loss of *N*-acetylhexosamine by destruction, as estimated from a control. The control contained an amount of free *N*-acetylgalactosamine equivalent to the hexosamine content of the glycopeptide and was treated under the same conditions but only for 2/3 of the time. The detailed analysis of the experiment is given in Table V. As may be seen from the table, after 48 h the release of 1518 μmoles of *N*-acetylgalactosamine coincided with the loss of 1527 μmoles of serine plus threonine; the concentration of the other amino acids did not change significantly. No correction was applied for the contribution to the loss of serine and threonine made by the destruction of serine and threonine residues with a free hydroxyl group because of the small amount of such residues in the glycopeptides. The losses of serine in L-Ser–L-Leu–L-Leu and in Gly–L-Ser, treated with 0.5 N NaOH for 72 h at 0°, were 4.7 and 5.7%, respectively. The time curve of the release of *N*-acetylhexosamine from glycopeptide preparation 17 is shown in Fig. 3.

TABLE V

EFFECT OF ALKALI TREATMENT ON COMPOSITION OF GLYCOPEPTIDE PREPARATION No. 17

Results expressed in μmoles/gram glycopeptide. Conditions: 0.5 N NaOH; 22°.

| *Time (h)* | *Free N-acetyl-galactos-amine* | *Ser* | *Thr* | *Gly* | *Pro* | *Ala* | *Val* | *Leu* | *Glu* | *Asp* | *Lys* | *Arg* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 1241 | 1164 | 1323 | 754 | 749 | 400 | 179 | 236 | 92 | trace | trace |
| 48 | 1518 | 562 | 316 | 1257 | 702 | 710 | 392 | 150 | 251 | 72 | — | — |
| Change | +1518 | −679 | −848 | −66 | −52 | −39 | −8 | −29 | +15 | −20 | — | — |
| | | | −1527 | | | | | | | | | |

On treatment of glycopeptides with 0.5 N NaOH at room temperature an increase in the absorbancy at 241 mμ was observed. Fig. 4 shows this increase with time when preparation 18 was treated as above. The experiment was recorded

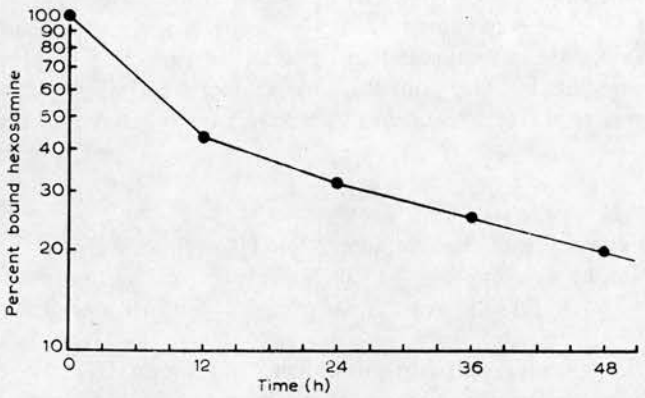



Fig. 3. Logarithmic plot of decrease of bound hexosamine as function of time on alkali treatment of glycopeptide (0.5 N NaOH; 22°).

automatically in a Zeiss recording spectrophotometer Model RPQ 20 A V and the absorbancies at 241 mμ, at 5-min intervals, were plotted. *N*-acetylgalactosamine, exposed to the same conditions, did not disclose any change in absorbancy.

*Alkali treatment of OSM and analysis of the reaction products*

As is evident from Table I, the recovery of total hexosamine after treatment of OSM with pronase at pH 8.0 was only 81.3%, whereas the sialic acid was recovered nearly quantitatively. Experiments were carried out to investigate the effect of alkaline treatment alone, *i.e.* in the absence of pronase, on OSM.

58.7 mg of OSM was dissolved in 10.0 ml of 0.01 M $CaCl_2$; 9.0 ml of this solution (containing 52.8 mg OSM) was adjusted to pH 8.0 and incubated for 5 days at 42° in the presence of toluene, the remainder being used for analysis of the untreated OSM. The pH was kept constant by re-adjustment from time to time. At

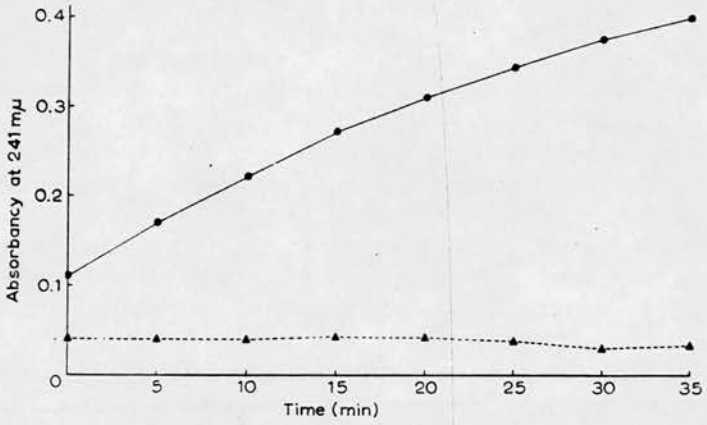



Fig. 4. Change in absorbancy at 241 mμ with time when glycopeptide preparation No. 18 and *N*-acetylgalactosamine respectively were treated with alkali at room temperature. Assay (●—●): 116 μg glycopeptide per ml 0.5 N NaOH. Control (▲---▲): 50 μg *N*-acetylgalactosamine per ml 0.5 N NaOH. The compounds, dissolved in a small volume of water, were added to the alkali in the cuvettes at time 0. The absorbancy at 241 mμ was recorded with a Carl Zeiss recording spectrophotometer Model RPQ 20A V using cuvettes of 0.5-cm light path.

the end of this treatment the volume was made up to 10.0 ml and aliquots were analysed for material reacting in the Morgan–Elson test, for total hexosamine (after standard acid treatment) and for free and total *N*-acetylneuraminic acid. The residual volume (9.0 ml) was dialysed against distilled water (300 ml) with stirring at 4° for 96 h in the presence of toluene. The bag content and the concentrated dialysate were analysed for the same substances as described above for the unfractionated assay. The results are presented in Table VI. This table shows that in the unfractionated assay *N*-acetylneuraminic acid was recovered quantitatively. Even on prolonged dialysis the substance(s) reacting in the WARREN test did not pass through the wall of the dialysing bag, whereas from an OSM–neuraminidase digest 92.1% of

TABLE VI

CARBOHYDRATE ANALYSIS OF ALKALI-TREATED OSM BEFORE AND AFTER DIALYSIS

Conditions: pH 8.0, 42°, 120 h. The experimental figures are expressed as μmoles/52.8 mg OSM; the figures are not corrected for the moisture content of lyophilized OSM.

| | *Native OSM* | *OSM after alkali treatment* | | |
|---|---|---|---|---|
| | | *Unfractioned assay* | *Dialysed assay* | |
| | | | *Retentate* | *Dialysate* |
| WARREN[11] value** | 0.63 | 8.42 | 8.75 | 0.69 |
| Total NANA | 35.49 | 35.89 | 27.00 | 8.91 |
| Percent recovery of NANA | — | 101.1 | 76.1 | 25.1 |
| Morgan–Elson value* | 0.39 | 6.80 | 0.12 | 6.00 |
| Hexosamine | 36.06 | 29.66 | 22.93 | 5.70 |
| Per cent recovery of hexosamine | — | 82.3 | 63.6 | 15.8 |

* Expressed as *N*-acetylgalactosamine.
** Expressed as NANA.

the liberated *N*-acetylneuraminic acid passed into the dialysate within 48 h. When an aliquot of the retentate was treated with 0.1 N $H_2SO_4$ at 80° for 60 min, the WARREN test[11] gave a value of 27.3 μmoles (expressed as NANA), as compared with 27.0 μmoles for the non-hydrolysed retentate by the resorcinol method (see Table VI). A similar figure for the *N*-acetylneuraminic acid content of the non-hydrolysed retentate was obtained by the direct Ehrlich reaction.

Hexosamine was recovered only to the extent of 82.3%, thus establishing that the loss of 18.7% of hexosamine recorded in Table I can be accounted for solely by the effect of alkali on OSM. 63.6% of the total hexosamine was recovered as bound hexosamine in the retentate. That, in fact, all hexosamine present in the retentate was bound in a glycosidic linkage, was suggested by the minute amount of material reacting in the Morgan–Elson test. It was also shown that enzymatic removal of sialic acid from the alkali-treated OSM prior to dialysis did not change the distribution of hexosamine. In this case 63% of the total hexosamine was found in the retentate. It would appear, therefore, that in the experiment of Table VI 36.4% of the total hexosamine was released by the mild alkali treatment.

In the dialysate 5.70 μmoles of hexosamine, after acid treatment, were recovered, *i.e.* 15.8% of the total hexosamine and 43.4% of the hexosamine released on alkali treatment of OSM. When, however, the dialysate was submitted to the Morgan–Elson reaction, 6.00 μmoles of material reacting as *N*-acetylhexosamine and expressed as such were found, *i.e.* 88.2% of the same material present in the unfractionated assay. This material represents 16.6% of the total *N*-acetylhexosamine and 45.7% of the *N*-acetylhexosamine released from OSM upon mild alkali treatment. Chemically it is most likely a mixture of *N*-acetylhexosamine (substituted at C-6 by NANA) and chromogens I and III (substituted at C-6 by NANA). This is suggested by the observation that the dialysate reacted not only in the Morgan–Elson reaction but also produced a purplish colour on addition of Ehrlich's reagent in the cold. This colour was unstable to heating and to mineral acid. The 6-substituted chromogens I and III are known to be formed from the free prosthetic groups (*N*-acetylneuraminyl (2 → 6) *N*-acetylgalactosamine) by loss of 1 and 2 molecules of $H_2O$, respectively, on mild alkali treatment and they are characterized by the above properties[1,20].

51% of the total hexosamine released from OSM on alkali treatment was degraded to such a degree that it reacted neither in the Morgan–Elson nor in the Elson–Morgan tests. In a model experiment in which 18.73 μmoles of *N*-acetylgalactosamine were treated at pH 8.0 and 42° for 120 h, 12.89 μmoles of material reacting in the Morgan–Elson procedure and expressed as *N*-acetylhexosamine were recovered, *i.e.* 68.8% of the total. The alkali-treated *N*-acetylgalactosamine gave a purple colour with Ehrlich's reagent in the cold. When the alkali-treated *N*-acetylgalactosamine was heated with 2 N HCl at 100° for 4 h, 10.56 μmoles of hexosamine, *i.e.* 56.4% of the theoretical were recovered.

*Methoxyneuraminic acid in the thiobarbituric acid and resorcinol tests*

109.2 μg (= 0.389 μmoles) of crystalline methoxyneuraminic acid in 0.2 ml water and 11.05 μg (= 0.036 μmoles) of crystalline *N*-acetylneuraminic acid were submitted separately to the WARREN test[11]. The absorbancies, measured at 549 mμ in cuvettes of 1-cm light path in a Zeiss Spectrophotometer PMQ II, were 0.428 and

0.468, respectively. The molecular extinction coefficient of methoxyneuraminic acid is therefore only 8.5% of that of *N*-acetylneuraminic acid.

36.0 $\mu$g (= 0.128 $\mu$moles) of crystalline methoxyneuraminic acid in 2.0 ml water and 33.2 $\mu$g (= 0.107 $\mu$moles) of crystalline *N*-acetylneuraminic acid in 2.0 ml water were submitted separately to the resorcinol procedure of SVENNERHOLM[10]. The absorbancies, determined at 580 m$\mu$ as above, were 0.251 and 0.169, respectively, *i.e.* mole per mole methoxyneuraminic acid gives 24.3% more colour than *N*-acetylneuraminic acid.

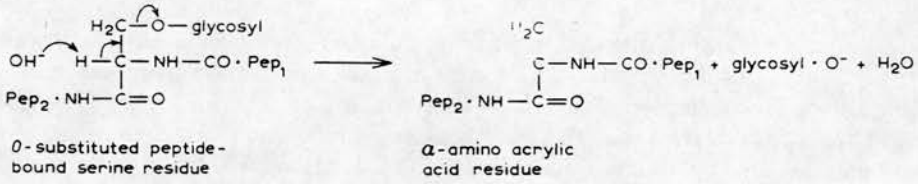
## DISCUSSION

From the data presented it would appear that digestion of OSM with pronase over a period of 120 h, followed by neuraminidase treatment, resulted in the formation of glycopeptides with an average content of four amino acid residues to which about 1.3 residues of *N*-acetylgalactosamine were attached. Quantitative amino acid analysis of the glycopeptides (Table II) revealed hydroxyamino acids as the only amino acids with a functional group in the side chain present in a total concentration high enough to accommodate all *N*-acetylgalactosamine residues. It is also evident from Table II when taken together with the average molecular weight of the preparations 15 and 17 (see Table III) that each preparation is a fraction consisting of glycopeptides of varying composition with serine and threonine as the predominant amino acids. The heterogeneity of the glycopeptide preparation is also seen in the electrophoretograms; this heterogeneity is to be expected from the random action of pronase on a polypeptide chain along which several hundred prosthetic groups are distributed. Proline and glycine prevailed among the amino acids without a functional group in the side chain. This finding may be due to the fact that certain peptide bonds, especially those involving glycine and proline (L-Pro–L-Leu, Gly–L-Pro, Gly–Gly), are resistant to pronase[21,22]. The low aspartic and glutamic acid content of the glycopeptides is accounted for by the presence in pool C (see RESULTS) of 58.3% of the total 156.2 $\mu$moles of dicarboxylic amino acids present in 250 mg OSM and by retention on the Dowex-1 column of glycopeptides containing 19.1% of the total dicarboxylic acids. Concerning the 0.11 and 0.18 moles, respectively, of monoamino dicarboxylic acids present in preparations 15 and 17 per mole galactosamine, their presence is probably due to the formation of the calcium salts of their free carboxyl groups during the enzymic digestions in a calcium medium. The low degree of dissociation of the calcium salts may have allowed some glycopeptides containing dicarboxylic acids to pass the Dowex-1 column (fourth step of preparation).

Treatment of the glycopeptides with 0.5 N NaOH resulted in the liberation of *N*-acetylgalactosamine and a concomitant loss of an approximately equimolar quantity of $\alpha$-amino-$\beta$-hydroxycarboxylic acids. This equivalence prevailed in experiments carried out at 0° and at 22°. Though at 22° more of the released amino sugar was destroyed than at 0°, 80% of the total hexosamine was liberated under this condition within a relatively short time (48 h). The slope of the decrease of bound hexosamine (see Fig. 3) would indicate that with the release of 80% of the total hexosamine the reaction had not yet reached the end-point. It seems therefore

likely that the residual 20% of hexosamine would also be split off with a concomitant loss of serine and/or threonine. Since the glycopeptides contained 50% of the total hexosamine of OSM, it would appear that at least 50% of the prosthetic groups are linked O-glycosidically to the hydroxyl groups of serine and threonine residues.

As to the mechanism of the reaction, the observations made by TANAKA *et al.*[6] and HARBON *et al.*[8] (see INTRODUCTION) strongly suggest that the liberation of *N*-acetylhexosamine proceeds by $\beta$-carbonyl elimination resulting in the formation of peptide-bonded $\alpha$-aminoacrylic acid (from serine) and $\alpha$-aminocrotonic acid or isomer (from threonine):




The resulting dehydropeptide, on acid hydrolysis with 6 N HCl at 110° for 24 h, will yield free amino acids and in addition pyruvic acid instead of serine and $\alpha$-ketobutyric acid instead of threonine. Our finding of the increase in absorbancy at 241 m$\mu$ on alkali treatment of the glycopeptides would be consistent with this mechanism, since the strong absorbancy at 241 m$\mu$ of $\alpha$-amino acrylic acid derivatives has been previously[23] reported.

As may be seen from Fig. 3, after the release of 56% of *N*-acetylgalactosamine in 12 h the rate of $\beta$-elimination decreased markedly. This decrease may result from the terminal positioning of some of the O-substituted seryl and threonyl residues. When in a C-terminal position, the carboxylate anion of an O-substituted seryl or threonyl residue will discourage $\beta$-elimination because of its mesomeric effect on the $\alpha$-carbon atom. When in an N-terminal position, the inductive effect of the non-protonated amino group will hinder the expulsion of the proton in $\alpha$-position.

On keeping native OSM at pH 8.0 and 42° for 120 h 36% of the total *N*-acetylhexosamine was released, half of which was degraded to such an extent that it did not react with the Ehrlich reagent under the conditions of the Morgan–Elson and Elson–Morgan tests. It was an unexpected but reproducible finding that the retentate of the dialysed reaction mixture contained 8.75 $\mu$moles of material reacting in the WARREN test[11] and expressed as NANA (Table VI). When the retentate was hydrolysed (0.1 N $H_2SO_4$, 80°, 60 min) to liberate the bound NANA, the Warren value of the hydrolysate (27.3 $\mu$moles) was practically the same as the resorcinol value (27.0 $\mu$moles) of the retentate before hydrolysis, indicating that part of the bound, non-dialysable NANA reacted in the WARREN test[11]. Since the chromogen in the WARREN test is formylpyruvic acid[24], which is formed only from free *N*-acylneuraminic acids and from *N*-acetyl-4-*O*-acetylneuraminic acid, the mild alkali treatment of OSM has apparently changed some of the bound NANA residues in such a way that the conditions of the WARREN procedure sufficed to cleave the ketosidic linkage. Assuming deacetylation of part of the NANA residues as the alkali-induced change, the action of periodate on the changed prosthetic groups

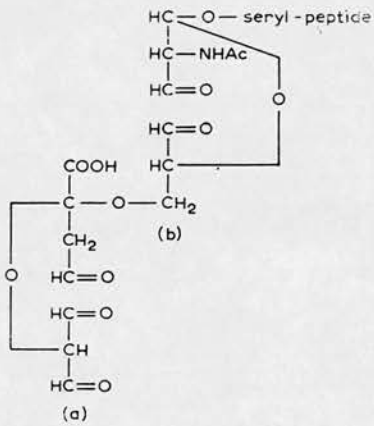



Fig. 5. Structure resulting from periodate oxidation of deacetylated NANA linked ketosidically to C-6 of a glycosidically-linked *N*-acetylgalactosamine residue.

would result in the structure shown in Fig. 5. This structure contains formylpyruvic acid in a ketosidic linkage rendered labile by the presence of carbonyl groups in $\beta$-position to both C-atoms involved in the ketosidic linkage. It is true that methoxyneuraminic acid, which on periodate oxidation will also give rise to the glycon structure (a) shown in Fig. 5, has only $^1/_{12}$th of the molecular extinction coefficient of NANA. However, the difference in the oxidized compounds is that in the case of methoxyneuraminic acid only the glycosidic carbon atom of the glycon (a) has a carbonyl group in $\beta$-position, whereas in the case of the prosthetic group both C-atoms involved in the glycosidic linkage are activated by $\beta$-carbonyl groups.

Some evidence for the assumed deacetylation of part of the NANA residues during digestion at pH 8.0 seems to be provided by the resorcinol value (expressed as NANA) of the retentate (see Table VI). Since in the native OSM the molar ratio NANA/hexosamine was 0.98, the same ratio may be expected for the isolated glycoprotein after release of about one-third of the prosthetic groups on keeping OSM at pH 8 and 42°. The observed value of this ratio was, however, 1.18. This figure would be consistent with a partial deacetylation of the NANA residues; for it was found that deacetylated NANA, as present in methoxyneuraminic acid, gives mole per mole 24.3% more colour in the resorcinol test than NANA.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. P. ANDERS, Max-Planck-Institut for Virusforschung, Abteilung Schramm, for the quantitative amino acid analyses and the Director of the Abattoir of the City of Stuttgart for the very helpful co-operation in obtaining fresh submaxillary glands from sheep. We are also grateful to Professor H. FAILLARD for his gift of crystalline methoxyneuraminic acid. This work has been supported in part by grants GM 10917-02 and AI 04954-02 of the U.S. Public Health Service.

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*Biochimica et Biophysica Acta*
Elsevier Publishing Company
Amsterdam
Printed in The Netherlands



BBA 85051

# STUDIES ON GLYCOPROTEINS

## XII. ABOUT GLYCOSIDIC-ESTER LINKAGES IN SUBMAXILLARY GLAND GLYCOPROTEINS PREPARED FROM SHEEP

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(Received January 18th, 1965)

SUMMARY

OSM prepared from sheep is known to contain O-glycosidic linkages involving the reducing group of *N*-acetylgalactosamine and the hydroxyl groups of serine and threonine. It is the aim of this paper to establish whether or not in addition to this linkage another type of alkali-labile linkage, in particular a glycosidic-ester linkage, is present in OSM.

In the first set of experiments it was found that the release of 35.2% of *N*-acetylgalactosamine, when OSM was kept at pH 8.0 and 42° for 120 h, was matched by the loss of an equimolar amount of serine and threonine without a significant change in the other amino acids. In a second set of experiments it was shown that on treating OSM with 0.1 N NaOH at 4° the decrease of bound hexosamine with time was linear. Finally, glycopeptides prepared by trypsin (EC 3.4.4.4) or pronase action on OSM were treated with $LiBH_4$ in tetrahydrofuran. No significant difference in the dicarboxylic amino acids before and after such treatment was observed. Only traces of homoserine and $\alpha$-amino-$\delta$-hydroxy-*n*-valeric acid were detectable after $LiBH_4$ reduction.

Since the $LiBH_4$ treatment of trypsinized OSM prepared from Australian sheep resulted in the loss of 35% of the dicarboxylic acid content, the suggestion is made that the fine structure of the ovine submaxillary glycoproteins may vary with the age of the animals. In Australia, lambs of an average age of 4 months were used for the work; in Europe only sheep of three years or more are slaughtered. Similar differences with age have been described for the fine structure of the chondroitin sulphates composing human hyaline cartilage.

## INTRODUCTION

In previous papers[1,2] it was shown that in OSM prepared from German sheep at least 50% of the prosthetic groups (*N*-acetylneuraminyl (2→6) *N*-acetylgalactos-

Abbreviations: OSM and BSM, ovine and bovine submaxillary-gland glycoprotein respectively; NANA, *N*-acetylneuraminic acid.

amine) are linked O-glycosidically to the hydroxyl groups of serine and threonine residues. The evidence for this type of linkage was that treatment with 0.5 N NaOH at 0° for 96 h or at 22° for 24 h effected the release from short glycopeptides, derived by pronase digestion of OSM, of two-thirds of the bound *N*-acetylhexosamine concomitant with the loss of an equimolar amount of $\alpha$-amino-$\beta$-hydroxycarboxylic acids, most probably by the mechanism of $\beta$-elimination. Shortly afterwards and independently, HARBON *et al.*[3] reported similar results. It was rather surprising to find that on keeping native sheep OSM at pH 8.0 and 42° for 120 h, about 36% of the total prosthetic groups were liberated[2]. Since the susceptibility to such mild alkaline conditions was suggestive of the existence in OSM of a second type of carbohydrate–peptide linkage still more sensitive to alkali than the O-glycosidic linkages to serine and threonine, three sets of experiments were performed. Firstly, native OSM was kept at pH 8.0 and 42° until about 35% of the prosthetic groups were released. At this stage a quantitative analysis of the hexosamine still bound to the protein and of the component amino acids was carried out and compared with the hexosamine and amino acid analyses of the control (untreated) OSM. Secondly, OSM was dissolved in 0.1 N NaOH containing 0.3 M sodium borohydride and kept at 4° for various periods of time; the decrease of bound NANA and hexosamine was determined. When these conditions were applied to BSM, the kinetics of the decrease of bound hexosamine indicated the presence of two different types of alkali-labile linkages[4]. Thirdly, for the detection of any glycosidic-ester linkages previously reported to be present in OSM from Australian lambs[5], glycopeptides prepared from submaxillary glands of sheep were treated with lithium borohydride in tetrahydrofuran and their dicarboxylic acid content was determined before and after such treatment. In these experiments a search was also made for homoserine and $\alpha$-amino-$\delta$-hydroxy-*n*-valeric acid which would arise from the reduction of esterified $\beta$- and $\gamma$-carboxyl groups respectively of aspartyl and glutamyl residues.

## EXPERIMENTAL

### *Materials*

OSM was prepared from sheep as described previously[2]. Dry methanol was prepared from absolute methanol as described by VOGEL[6]. Methanolic hydrochloric acid was prepared as described previously[5]. Tetrahydrofuran was purified and dried according to CRAWHALL AND ELLIOTT[7]. Lithium borohydride was purified according to CHIBNALL AND REES[8]. Crystalline homoserine was supplied by Sigma Chemical Company, St. Louis, U.S.A. Trypsin (EC 3.4.4.4), twice recrystallized, was supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks, England. Pronase was a preparation supplied by Sigma Chemical Company, St. Louis, U.S.A.

Cellulose casings (6 cm circumference) for dialysis were obtained from the Visking Corporation, Chicago. All glassware used for the reduction with lithium borohydride was dried at 105° for at least 12 h prior to use.

Resins used were Dowex-1 X4 (50–100 mesh) and Dowex-50W X4 (50–100 mesh). Sephadex G-25 was a preparation of Pharmacia, Uppsala, Sweden.

*Methods*

Measurements of pH were made with a Radiometer type TTT 1b equipped with a glass electrode type G 222 C.

*N*-Acetylneuraminic acid was determined by the resorcinol method of SVENNERHOLM[9] using crystalline NANA as standard. *N*-Acetylhexosamine was determined according to Morgan–Elson as modified by REISSIG, STROMINGER AND LELOIR[10]. Crystalline *N*-acetyl-D-galactosamine was used as standard.

Hexosamine was determined as described previously[11]. Prior to assay the material was hydrolysed with 3 N HCl at 100° for 8 h in a sealed tube. The hydrolysate was dried *in vacuo*. On the basis of model experiments a correction for the destruction of 10% galactosamine was applied. As standard crystalline D-galactosamine · HCl was used.

Protein was determined by the Lowry–Folin method[12] using crystalline serum albumin as standard.

The quantitative analysis of the individual amino acids in OSM hydrolysates was carried out with an amino acid analyser, Spinco Model 120.

In the $LiBH_4$ experiments the dicarboxylic acids were separated on a Dowex-1 (acetate form) column from the neutral and basic amino acids and determined with the ninhydrin reagent of MOORE AND STEIN[13]. The fraction containing the neutral and basic amino acids was the material assayed for homoserine and α-amino-δ-hydroxy-*n*-valeric acid.

For the quantitation of homoserine and α-amino-δ-hydroxy-*n*-valeric acid the same Beckman automatic analyser and the same columns and resins were used as for the estimation of the ordinary amino acids. However, the temperature was lowered to 28°; the buffer used was the usual 0.2 N sodium citrate (pH 3.25) and the rate of elution was 40 ml buffer per h. Under these conditions a good separation of homoserine from serine and of α-amino-δ-hydroxy-*n*-valeric acid from proline and glutamic acid was achieved. Homoserine appeared 14 ml and α-amino-δ-hydroxy-*n*-valeric acid 26 ml behind serine. This separation allows a convenient quantitative evaluation of homoserine and α-amino-δ-hydroxy-*n*-valeric acid, the valley between their peaks going down to 0.08 absorbance.

Paper chromatography in various solvents was carried out as described previously[2].

The reducing power of the stock solution of lithium borohydride in tetrahydrofuran was estimated prior to each reduction by the method of LYTTLE, JENSEN AND STRUCK[14].

The reduction by $LiBH_4$ in tetrahydrofuran of glycopeptides prepared from OSM as detailed under RESULTS was carried out in a 50-ml conical flask, fitted with a three-neck adaptor. One neck bore a Liebig condenser fitted with a $CaCl_2$ drying-tube, another a thermometer and the third one was stoppered, being used for the addition of the reactants. The flask was heated on a hotplate and the content of the flask was stirred magnetically. The reduction procedure, the decomposition of excess $LiBH_4$, the hydrolysis of the reduction products and the separation of the monocarboxylic amino acids from the dicarboxylic amino acids on a Dowex-1 (acetate form) column have been described previously[5].

*Synthesis of α-amino-δ-hydroxy-n-valeric acid*

γ-Methyl glutamate hydrochloride was prepared according to COLEMAN[15],

except that methanol was used instead of ethanol; m.p. 152–153° (uncorr.). The ester was reduced under the conditions used by MURPHY AND GOTTSCHALK[16]. The α-amino-δ-hydroxy-*n*-valeric acid had a melting point of 228–229° (uncorr.).

## RESULTS

*Effect of mild alkali treatment at 42° on the carbohydrate and protein moieties of OSM*

About 100 mg of OSM was dissolved in 20.0 ml of 0.01 M $CaCl_2$; 18.0 ml of this solution was adjusted to pH 8.0 and incubated at 42° in the presence of toluene; the remainder was kept frozen until used as control. The pH was kept constant by readjustment from time to time. Aliquots were withdrawn every 48 h, adjusted to pH 6.0 with 0.1 N HCl and the progress of the reaction, *i.e.* the release of prosthetic groups, was measured by the Morgan–Elson reaction. As was shown in the previous paper[2], about half of the *N*-acetylgalactosamine of the released prosthetic groups was destroyed under the same conditions in 120 h.

When the Morgan–Elson reaction in the assay indicated the release of about 35% of the total prosthetic groups, the experiment was interrupted by adjusting the pH to about 6.0. One ml of the assay and 1.0 ml of the control were treated with 6 N HCl for 24 h at 110° in an atmosphere of nitrogen in sealed tubes. The hydrolysates were dried in a vacuum-desiccator, dissolved in water, filtered (to remove humin) and the filtrate plus washings were dried. The dry residue was dissolved in 4.0 ml of 0.2 M sodium citrate buffer (pH 2.2) and an aliquot (1.0 ml) was analysed for neutral and acidic amino acids.

10.0 ml of the neutralized assay (see above) was exhaustively dialysed, with stirring, against de-ionized water at 4° in the presence of toluene. The content of the bag was made up to 25.0 ml and a 2.0-ml aliquot was analysed for hexosamine after acid hydrolysis.

In order to ascertain that no bound hexosamine was lost on dialysis of the assay, the dialysate was concentrated to a small volume and submitted to paper chromatography. When the chromatograms were sprayed for the detection of glycopeptides with periodate–benzidine and peptide sprays, a weak spot at the base line reacting with both reagents was seen, indicating the presence in the dialysate of some glycopeptide material. This did not amount to more than 2.0% of the OSM, as judged from the colour intensity of the periodate–benzidine spot.

The results of the analyses are shown in Table I.

*Effect of alkali treatment at 4° on the carbohydrate moiety of OSM, using the conditions of* TANAKA, BERTOLINI AND PIGMAN[4]

About 150 mg of OSM was dissolved with shaking in 30.0 ml of 0.1 N NaOH containing 0.3 M sodium borohydride; a 5.0-ml aliquot was withdrawn and used as control. The remainder was kept at 4° and 5.0-ml aliquots were removed after various time intervals. Assays and control were neutralized and dialysed as described by TANAKA, BERTOLINI AND PIGMAN[4]. The content of the bag was made up to 25.0 ml and aliquots were analysed for NANA, hexosamine and total protein. The results are shown in Table II. Since the protein content of the retentates of the assays was not significantly different from that of the retentate of the control, no correction was applied to the carbohydrate figures.

TABLE I

AMINO ACID AND HEXOSAMINE ANALYSES OF SHEEP OSM BEFORE AND AFTER TREATMENT WITH ALKALI (pH 8.0 AND 42°)

Results are expressed in μmoles/96.8 mg OSM.

| *Time (h)* | *Bound galactosamine* | *Ser* | *Thr* | *Gly* | *Pro* | *Ala* | *Val* | *Leu* | *Ileu* | *Phe* | *Tyr* | *Asp* | *Glu* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 64.2 | 62.8 | 50.8 | 70.4 | 30.0 | 44.4 | 29.1 | 24.0 | 12.6 | 12.1 | 8.6 | 23.3 | 27.4 |
| 120 | 41.6 | 48.7 | 41.8 | 70.4 | 30.7 | 46.6 | 29.3 | 25.8 | 13.1 | 12.0 | 10.2 | 24.8 | 24.8 |
| Change | −22.6 | −14.1 | −9.0 | 0.0 | +0.7 | +2.2 | +0.2 | +1.8 | +0.5 | −0.1 | +1.6 | +1.5 | −2.6 |
| | | −23.1 | | | | | | | | | | | |

*Effect of $LiBH_4$ treatment on the dicarboxylic amino acid content of enzymatically fragmentated OSM*

A. About 50 mg of OSM, dissolved in 10.0 ml of 0.1 M phosphate buffer (pH 8.0) was digested with 1 mg of crystalline trypsin for 16 h at 37° (in the presence of toluene). The digest was dialysed against distilled water (saturated with toluene) at 4° for 72 h. The retentate was made up to 50.0 ml volume. Two aliquots of 22.0 ml each were transferred to the reduction flasks (see METHODS) and dried in a vacuum-desiccator.

TABLE II

DECREASE OF BOUND NANA AND HEXOSAMINE OF SHEEP OSM AFTER TREATMENT WITH 0.1 N NaOH–0.3 M $NaBH_4$ AT 4°

Results are expressed in mg/154.5 mg OSM.

| | *Time of treatment in hours* | | | | |
|---|---|---|---|---|---|
| | *0* | *24* | *72* | *144* | *216* |
| Protein | 88.5 | 90.3 | 89.1 | 89.6 | 85.6 |
| NANA | 25.8 | 24.1 | 21.2 | 18.2 | 15.3 |
| Hexosamine* | 19.5 | 18.3 | 15.6 | 13.2 | 10.7 |

* Expressed as D-galactosamine · HCl.

To one flask was added 15 ml of 0.3 M $LiBH_4$ in tetrahydrofuran and the solution was refluxed for 6 h. The reaction mixture was then stirred with about 2 ml of 2 N methanolic hydrochloric acid for 1 h at 0° and taken to dryness in a desiccator. Residual borate was removed by repeated evaporation from methanolic hydrochloric acid. The dry residue was hydrolysed in 5 ml of 6 N HCl at 110° for 24 h in a sealed tube. After removal of the hydrochloric acid (see above), the dicarboxylic acids were separated on a Dowex-1 (acetate) column, eluted with 1 N acetic acid and quantitated according to MOORE AND STEIN[13]. The other aliquot of 22.0 ml was treated in the same manner except that $LiBH_4$ was omitted.

TABLE III

THE DICARBOXYLIC AMINO ACID CONTENT OF GLYCOPEPTIDES FROM SHEEP OSM BEFORE AND AFTER TREATMENT WITH $LiBH_4$

Results are expressed in μmoles and refer to 50.0 mg of parent OSM.

| *Preparation of glycopeptides from native sheep OSM** | *Time of refluxing with $LiBH_4$ in tetrahydrofuran (h)* | *Dicarboxylic acid content* | |
|---|---|---|---|
| | | *before treatment* | *after treatment* |
| Trypsin, pH 8.0, 37°, 16 h; dialysis | 6 | 28.0 | 29.6 |
| Trypsin, pH 8.0, 37°, 16 h; dialysis | 6 | 35.6 | 36.3 |
| Pronase, pH 7.0, 37°, 24 h; fractionation on Sephadex G-25 | 5 | 7.5 | 8.7 |
| Pronase, pH 8.0, 42°, 120 h; fractionation on Sephadex G-25 | 8 | 8.2 | 7.0 |
| Pronase, pH 8.0, 37°, 72 h; not fractionated | 10 | 32.9 | 31.2 |

* Not corrected for moisture.

The results of the experiments are shown in Table III.

B. About 50 mg of OSM was dissolved in 10.0 ml of 0.01 M $CaCl_2$ solution and the pH adjusted to 7.0. This mixture was digested with 2 mg of pronase for 24 h at 37°, the pH being kept constant. After concentration to a small volume the digest was applied to a Sephadex G-25 column and developed with distilled water. The fractionation of the digest was performed as described previously[2]. For the reduction experiments the sialoglycopeptide fraction (Pool B) was taken. Pool C (free amino acids and short peptides) gave a ninhydrin value equivalent to 81 μmoles of L-leucine indicating a considerable degree of fragmentation.

Pool B was concentrated to a volume of 10.0 ml. An aliquot of 5.0 ml was dried and submitted to the reduction and subsequent procedures as described under A. As control, the dicarboxylic acid content in an aliquot of the residual concentrate was determined.

In some experiments pronase digestion was carried out as in the previous paper[2] (pH 8.0, 42°, 120 h). In one experiment about 50 mg of OSM was treated with 3 additions of pronase (totalling 3.6 mg) at pH 8.0 and 37° for 72 h. In this case no fractionation of the digest prior to $LiBH_4$ treatment was undertaken; a corresponding amount of pronase was added to the control immediately before acid hydrolysis.

The results of the pronase experiments are also shown in Table III.

In the pronase experiment at pH 7.0 the glycopeptides, after treatment with $LiBH_4$, were assayed for homoserine and α-amino-δ-hydroxy-*n*-valeric acid. Only traces of these reduction products were found, their sum total being 0.06 μmole or even less, *i.e.* 0.8% or less of the total dicarboxylic acids present.

*Reduction by $LiBH_4$ of the model ester, γ-methyl glutamate*

To test the efficiency of the reduction procedure applied, 8.9 μmoles of the

model ester, $\gamma$-methyl glutamate, were submitted to all steps of the procedure described under A. When the effluent and the eluate from Dowex-1 column were analysed for amino acids, the following results were obtained: effluent, 6.52 $\mu$moles; eluate, 2.48 $\mu$moles. It was found in an independent experiment that the colour value of $\alpha$-amino-$\delta$-hydroxy-$n$-valeric acid was 96% of that of the L-leucine standard. By paper chromatography, taking authentic $\alpha$-amino-$\delta$-hydroxy-$n$-valeric acid as reference substance, it was ascertained that the effluent contained only $\alpha$-amino-$\delta$-hydroxy-$n$-valeric acid and the eluate only glutamic acid. These figures would indicate a reduction of 73.3% of the ester.

## DISCUSSION

It was the aim of the experiments described in this paper to find out whether or not in the OSM preparation from German sheep in addition to the O-glycosidic linkages to serine and threonine known to be present[2,3] another type of alkali-labile linkage, in particular a glycosidic-ester linkage, occurs.

In the first set of experiments 35.2% of the total bound hexosamine was liberated when the OSM preparation was kept at pH 8.0 and 42° for 120 h. For each

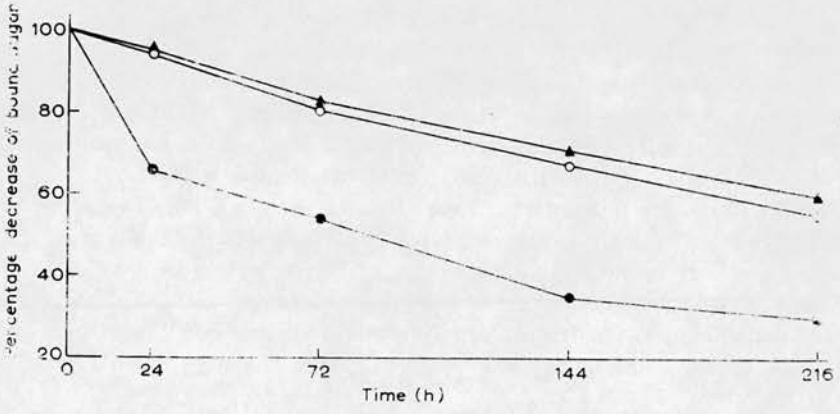


Fig. 1. Decrease with time of bound hexosamine and NANA (▲—▲) on treatment of OSM and BSM with 0.1 N NaOH at 4°. Hexosamine bound in sheep OSM (○—○), hexosamine bound in BSM (●—●).

mole of hexosamine released one mole of serine or threonine was lost (Table I). In the second set of experiments the decrease with time of bound hexosamine and NANA on treatment of OSM with 0.1 N NaOH–0.3 M $NaBH_4$ at 4° was determined (Table II). Under these conditions TANAKA, BERTOLINI AND PIGMAN[4] had observed for BSM a rapid fall of bound hexosamine during the first 24 h, followed by a slow decrease over the next 192 h. Fig. 1 shows on a comparable scale the results of TANAKA, BERTOLINI AND PIGMAN with BSM and our results with OSM. Whereas the time curve obtained with BSM displays a sharp decrease in gradient after 24 h, indicating the presence of two types of carbohydrate–peptide linkages differing in their degree of alkali lability, the time curves obtained with OSM are very close to a straight line. Moreover, in the

BSM preparation the release of hexosamine, on a molar basis, was more than the loss of serine plus threonine. Thus, after 144-h treatment 68.9 μmoles of hexosamine were liberated for a total loss of 51.5 μmoles of α-amino-β-hydroxycarboxylic acids, indicating that 17% of the total hexosamine residues are linked to the peptide by a bond other than a glycosidic linkage to serine and/or threonine. The high sensitivity of this other bond to alkali would be compatible with a glycosidic-ester linkage, as pointed out by TANAKA, BERTOLINI AND PIGMAN[4].

In the third set of experiments glycopeptides prepared from OSM by trypsin or pronase digestion were treated with $LiBH_4$ in tetrahydrofuran and the dicarboxylic acid content of the protein moiety was determined before and after this treatment. With the trypsin-pretreated OSM no significant difference was observed after 6-h $LiBH_4$ treatment. Also the small glycopeptides, resulting from pronase digestion of sheep OSM at pH 7.0, did not lose more than 10.7% of their dicarboxylic acids on treatment with $LiBH_4$ for 6 h, a loss too small to be regarded as significant. This is also evident from the negligible amount of homoserine and α-amino-δ-hydroxy-*n*-valeric acid formed in this experiment.

The efficiency of $LiBH_4$ to reduce esters under the conditions applied in the above experiments is shown by the reduction of 73% of γ-methyl glutamate to α-amino-δ-hydroxy-*n*-valeric acid. On the other hand, the recovery of 94.6% and 94.9% respectively of the dicarboxylic acids of glycopeptides (prepared from OSM by pronase action at pH 8.0 and known to contain only O-glycosidic linkages to serine and threonine[2]) on $LiBH_4$ treatment for 6 and 10 h would indicate that peptide-bonded aspartyl and glutamyl residues are not effected by this treatment. This is in agreement with out previous finding[16] that $LiBH_4$ treatment of poly-α-glutamic acid yielded on hydrolysis only 3.4% less free glutamic acid than did a control similarly treated, but omitting refluxing with $LiBH_4$ in tetrahydrofuran.

It would thus appear from the three approaches made that the existence in OSM (German sheep) of a glycosidic-ester linkage or any other linkage more alkali-labile than is the O-glycosidic linkage to serine or threonine can be excluded.

These results seem to contradict our previous observations with OSM prepared from Australian sheep. When trypsinized Australian OSM was submitted to $LiBH_4$ treatment under the conditions applied to German OSM, it lost 35% of its dicarboxylic acids in 7 h.

In a similar manner, the results of hydroxylamine treatment of OSM prepared from Australian and European sheep differ. Treatment of OSM (Australian sheep) with hydroxylamine at pH 12.2 and 37° resulted in the release of prosthetic groups concomitant with the formation of hydroxamates. The released disaccharides were quantitatively recovered, mainly in form of their oximes. Under similar conditions of alkalinity but in the absence of hydroxylamine, disaccharides were released at a much lower rate[11]. When HARBON, HERMAN-BOUSSIER AND CLAUSER[20] treated their European OSM at pH 12.8 and 50° for 60 min, the presence of hydroxylamine (1.6 M) did not alter the rate of release of the prosthetic groups.

Obviously then, the results obtained with Australian OSM and European OSM are consistent in themselves, but differ from each other. The suggestion is made that the difference in age of the sheep used for the material may be a contributing factor. At the Canberra abattoirs (from which the glands were collected for linkage studies) more than 80% of the sheep slaughtered are lambs at the age of 3 to 9 months

(average 4 months), the reason being that Canberra has a selected population without industrial workers. In Germany and France practically only adult sheep of three years or more are killed and the meat sold as mutton. There are also differences in breed, in pastures and other living conditions between Australian and European sheep.

KAPLAN AND MEYER[21] have shown that costal cartilage of newborn infants contains only chondroitin 4-sulphate, that of adults mainly chondroitin 6-sulphate. For human intervertebral discs BUDDECKE AND SZIEGOLEIT[22] found similar changes with age. It is also known[23] that the sialic acid of serum of 3–6-month-old calves consists of about 25% *N*-glycolylneuraminic acid and 75% *N*-acetylneuraminic acid (disregarding any O-acetylation). In ox serum the sialic acid contains about 65% *N*-glycolylneuraminic acid, the rest being the N-acetylated compound. For fetuin, the main serum protein of fetal calf, KLENK AND UHLENBRUCK[24] and SPIRO[25] have shown that *N*-glycolylneuraminic acid contributes but 5–7% to the total sialic acid content.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. P. ANDERS, Max-Planck-Institut für Virusforschung, Abteilung Schramm, for the quantitative amino acid analyses. We also much appreciate the useful discussions we had with Professor E. BUDDECKE, Department of Physiological Chemistry, University of Tübingen, and the helpful advice given to one of the authors (A.G.) by Professor W. H. STEIN, Rockefeller Institute, New York. This work has been supported by grant GM-10917-02 of the U.S. Public Health Service which we acknowledge with gratitude.

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Reprinted from Science, March 18, 1966, Vol. 151, No. 3716, pages 1404-1405

# Mucopolysaccharides: N-Acetylglucosamine- and Galactose-6-Sulfates from Keratosulfate

Abstract. *Galactose-6-sulfate and N-acetylglucosamine-6-sulfate were obtained pure from a partial acid hydrolyzate of corneal keratosulfate by paper chromatography and electrophoretic fractionation. These sugars were also present in hydrolyzates of skeletal keratosulfate. The distribution of the sulfate groups in the various keratosulfates might depend upon their source, and this distribution probably has important biological significance.*

Keratosulfate is a sulfated mucopolysaccharide composed of equimolar amounts of D-galactose and *N*-acetyl-D-glucosamine and of variable quantities of methylpentose and sialic acid (*1*). It has been isolated from cornea (*2*), nucleus pulposus (*3*), and cartilage of mammals and lower species (*4*). In these tissues keratosulfate is covalently bound to protein by at least two different types of bonds, either by *N*-glycosyl bonds to the amide groups of asparagine and glutamine or by *O*-glycosyl bonds to the hydroxyls of serine and threonine (*1*). The *O*-glycosidic bonds are alkali-labile and are split by $\beta$-elimination (*5*). The *N*-glycosyl bonds are mainly present in keratosulfate of cornea, the *O*-glycosyl linkages in skeletal keratosulfate. The structure of corneal keratosulfate was deduced from methylation and enzymatic digestion of the sulfated and desulfated polymer as *N*-acetyllactosamine-6-sulfate, polymerized by 1,3 bonding of the alternating hexosaminyl and D-galactosyl moieties (*6*).

Since the yields of the isolated methyl ethers were rather low, the methylation of bovine cornea keratosulfate was reinvestigated. Improved methods markedly increased the yields of methyl ethers, which, in general, confirmed the proposed structure. However, the evaluation of the experiment suggested that carbon No. 6 of galactose was partly blocked, indicating either branching at this position or substitution by another group.

After searching for optimum conditions we obtained, on hydrolysis of 1.0 g of keratosulfate for 1 hour with 0.5*N* $H_2SO_4$ at 100°C, two sulfated monosaccharides, which were isolated by paper chromatography and electrophoresis. Fraction I (8.0 mg) was galactose-6-sulfate, the other (fraction II, 13.2 mg) *N*-acetylglucosamine-6-sulfate. In addition, a number of sulfated oligosaccharides were present.

The properties of fraction I are as follows: $[\alpha]_D$ as barium salt was +42.5°, as ammonium salt was +52.0°. In paper chromatography its migration was 10 percent slower than that of synthetic glucose-6-sulfate. On paper electrophoresis at *p*H 6 it had the same mobility as glucose-6-sulfate. Its staining with aniline phthalate and tetrazolium chloride showed that the reducing group and carbon No. 2 were free.

The ratio of sulfate to galactose was 1.06. The reducing value (Schales and Schales) was 84 percent that of galactose. On infrared spectroscopy the substance absorbed at 1240 and 820 $cm^{-1}$, showing the absence of a sulfate on carbon No. 4. It consumed 3 moles of periodate rapidly in unbuffered solution and then consumed slowly an additional mole. Negligible amounts of formaldehyde were released on oxidation in bicarbonate buffer (*p*H 7.5) for 24 hours (*7*).

The properties of fraction II were as follows: $[\alpha]_D$ as ammonium salt was +47.9°. Chromatographically and electrophoretically it had the same mobility as authentic *N*-acetylglucosamine-6-sulfate. It stained with aniline phthalate and gave a positive reaction for *N*-acetylhexosamine. The ninhydrin reaction was negative. On complete hydrolysis glucosamine was the only carbohydrate present. The ratio of sulfate to *N*-acetylglucosamine was 0.97, that of sulfate to glucosamine was 0.98. The reducing value (Schales and Schales) was 98 percent that of *N*-acetylglucosamine. On infrared spectroscopy the substance absorbed at 1240 and 820 $cm^{-1}$, indicating a primary sulfate ester group. On periodate oxidation in bicarbonate buffer it liberated formaldehyde only in traces.

The data strongly indicate that fraction I is galactose-6-sulfate and that fraction II is *N*-acetylglucosamine-6-sulfate. Paper chromatography and electrophoresis of a similarly hydrolyzed sample of keratosulfate from cartilage of human rib likewise yielded galactose-6-sulfate. Judged from the intensity of the spots, the content of galactose-6-sulfate in this fraction appears to be higher than that of keratosulfate of cornea. The isolation of *N*-acetylglucosamine-6-sulfate is in agreement with the structure previously proposed.

The presence of galactose-6-sulfate is another instance of the variations in the mucopolysaccharides in general and in keratosulfate in particular, and is another example of the apparent randomness of the biosynthesis of these compounds.

The isolation of galactose-6-sulfate from keratosulfate of cornea is noteworthy in that the ratio of sulfate to hexosamine in cornea is always close to one, while keratosulfate of senile human cartilage, as well as that of elasmobranch cartilage, is oversulfated; that is, the ratio of sulfate to hexosamine is greater than one (*1*). In these sources the extra sulfate can be expected to be in the galactosyl moiety. Galactose sulfate in mammalian tissue occurs in the sulfatides of brain and other tissues (*8*), and recently galactose-6-sulfate was demonstrated in neuraminlactose sulfate from the mammary glands of rats (*9*).

The finding of sulfate ester groups in both galactosyl and hexosaminyl moieties of keratosulfate may explain the anomalous behavior of this polymer toward quaternary ammonium and pyridinium salts (*10*), which may be caused by the proximity of sulfate groups in neighboring instead of in alternating glycosyl groups, so that the compound behaves similarly to heparin.

In view of our finding, we should not assume that the keratosulfate fractions that are isolated from different normal and abnormal tissues, such as degenerating cartilage, nucleus pulposus, and tissue in Marfan's syndrome, are identical.

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16 December 1965

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 242, No. 19, Issue of October 10, pp. 4352-4359, 1967
*Printed in U.S.A.*

# Studies on Keratosulfates

## METHYLATION AND PARTIAL ACID HYDROLYSIS OF BOVINE CORNEAL KERATOSULFATE*

(Received for publication, May 22, 1967)

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### SUMMARY

**Bovine corneal keratosulfate was permethylated in non-aqueous solvent after prior peracetylation. The methyl ethers were isolated after hydrolysis and characterized either as crystalline derivatives or by their physical and chemical properties. The main repeating unit is *N*-acetyllactosamine polymerized via a 1 → 3 linkage to galactose, as reported previously. About 74% of the *N*-acetylglucosaminyl groups are sulfated in position 6. However, about 40% of the galactosyl groups are also substituted in position 6, at least in the major portion by sulfate ester groups. This was shown by the isolation and characterization of galactose 6-sulfate from partial acid hydrolysates. In addition, *N*-acetylglucosamine 6-sulfate and oligosaccharides sulfated to varying degrees were also obtained. The polymer contained about 25% of galactosyl groups in excess of hexosamine. This excess is preferentially hydrolyzed and may indicate branch points. Evidence of *N*-methylation of glucosamine was obtained. The color reactions and nuclear magnetic resonance spectrum of *N*-methylglucosamine are compared with those of *N*-acetylglucosamine.**

The repeating unit of keratosulfate from bovine cornea was shown by methylation studies (1), to be (1 → 3)-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranosyl 6-sulfate. However, the yields in these experiments were not satisfactory, and the presence of 2,4-di-*O*-methyl-D-galactose in both methylated native and desulfated polysaccharide indicated either that the products were grossly undermethylated or that position 6 of galactose was blocked. It was therefore thought desirable to reinvestigate the methylation of KS I.[1] By carrying out the methylation in organic solvents it was possible to increase both the yield and the extent of methylation of the final product. Examination of the hydrolysate of this product showed the presence of 2,4-di-*O*-methyl-D-galactose, which comprised about 40% of the galactose derivatives. Once undermethylation or demethylation could be ruled out, it was concluded that about 40% of the galactose was blocked in position 6 either by branching or by sulfate groups. Lack of nonreducing end groups makes a major degree of branching improbable. This lack was shown by the total absence of 3,4,6-tri-*O*-methyl-D-glucosamine and the presence of only trace amounts of 2,3,4,6-tetra-*O*-methylgalactose and 2,3,4-tri-*O*-methyl-L-fucose in the hydrolysate of KS I. Further, the presence of sulfate groups on position 6 of galactose was confirmed by the isolation of galactose 6-sulfate from partial acid hydrolysates of KS I (2). A full report of this partial acid hydrolysis studies and the reinvestigation of the methylation of KS I is presented in this paper.

### EXPERIMENTAL PROCEDURE

*Materials*—Bovine corneas were obtained from Cudahy Company, Omaha, Nebraska. KS I was prepared and purified as described previously (4). The analytical values of the preparation were as follows (per cent of dry weight): hexose (anthrone method), 35.5; hexosamine, 26.1; uronic acid (carbazole method), 1.8; sulfate, 14.9; protein (Lowry), 10.3; methylpentose, 0.8; sialic acid (direct Ehrlich), 1.0; and $[\alpha]_D^{25}$ +3.0°.

*Sugars*—We gratefully acknowledge the following gifts of samples: 2,3,4-tri-*O*-methyl-*N*-phenyl-L-fucosylamine, 2,4,6-tri-*O*-methyl-D-galactose, and 2-acetamido-2-deoxy-3,4,6-*O*-trimethyl-D-glucose, from Professor R. Kuhn; 2,3,4,5-tetra-*O*-methyl- and 2,4,6-tri-*O*-methyl-*N*-phenyl-D-galactosylamines, and 2,3,4-tri- and 2,3,6-tri-*O*-methylgalactoses, from Professor G. O. Aspinall; 2,3,4,6-tetra-*O*-methyl-D-galactose, from Dr. K. O. Lloyd; 2,4-di-*O*-methyl-3,6-anhydro-D-galactose, from Dr. B. A. Lewis; and pentaacetyl-*N*-methyl-L-glucosamine, from Merck and Company, Inc.

*Analytical Methods*—These were the same as cited previously (3, 4) except for ester sulfate analysis, which was performed by the method of Antonopoulos (5).

Melting points were obtained on a microscope hot stage and are uncorrected.

Optical rotations in water solutions were measured with a

* This work was supported by a research grant from the United States Public Health Service.

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[1] The abbreviations used are: KS I, corneal keratosulfate; KS II, skeletal keratosulfate; NMR, nuclear magnetic resonance.

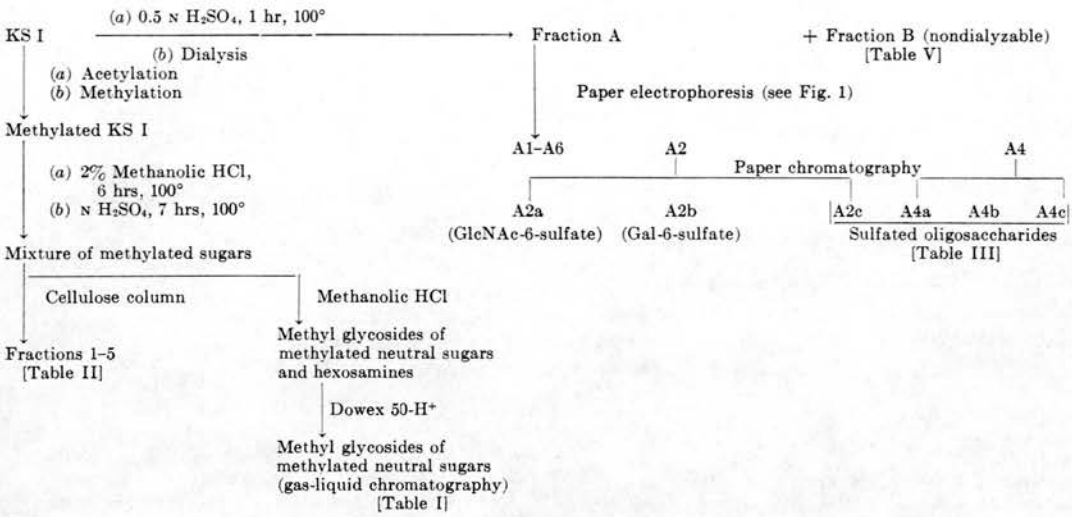


DIAGRAM 1. Methylation and partial acid hydrolysis of KS I

Bendix model 968 automatic polarimeter at room temperature, about 25°.

Infrared spectra were obtained with potassium bromide pellets in a Perkin-Elmer model 21 spectrophotometer with sodium chloride prism. All evaporations were carried out under reduced pressure and temperatures below 40°. Methylated sugars were demethylated with hydriodic acid (6) or with boron tribromide (7). Deionized water was used in all experiments on the isolation and characterization of sulfated sugars.

*Chromatography*—Paper chromatograms were developed by the descending technique on Whatman No. 1 paper. Whatman No. 3MM paper used for quantitative separation was previously washed with 0.01 N HCl, followed by distilled water, and finally with the solvent used in the separation. The following solvent systems (v/v) were used: A, butan-1-ol-ethanol-water (10:3:5); B, butan-1-ol-ethanol-water (4:1:5, upper layer); C, benzene-ethanol-water (169:47:15, upper layer); D, ethyl acetate-pyridine-water (10:4:3); E, ethyl acetate-acetic acid-water (6:3:2); F, ethyl acetate-pyridine-water (8:2:1); G, butan-1-ol-pyridine-water (5:3:2); and H, butan-1-ol-acetic acid-ethanol-water (10:3:1:7). Sugars were detected with aniline phthalate spray or with alkaline silver nitrate. Ninhydrin and Elson-Morgan sprays were used for hexosamines, and Morgan-Elson spray for N-acetylhexosamines. $R_G$ values of methylated sugars refer to rates of movement relative to 2,3,4,6-tetra-*O*-methyl-D-glucose in Solvent B.

Thin layer chromatography was carried out on plates coated with Silica Gel G or Silica Gel H according to Stahl (8). The plates were eluted by the ascending technique with the following solvent systems (v/v): (*a*) acetone-water-ammonia (250:3:1.5)[2] and (*b*) benzene-95% ethanol (20:5) (9). Sugars were detected with aniline phthalate spray.

Electrophoresis was performed on both Whatman No. 1 and prewashed Whatman No. 3MM paper (for quantitative separation) with the following buffer systems: I, 0.1 N acetic acid-pyridine, pH 5.5; II, 0.1 N acetic acid-pyridine, pH 3.5; and III, 0.05 N acetic acid-pyridine, pH 6.0. The same sprays described for paper chromatography were used for detection.

Cellulose columns were packed dry and washed extensively with water, followed by the solvent system used for elution until the washings gave a negative anthrone test. The solvents used for cellulose columns or for preparative separations on Whatman No. 3MM sheets were purified as follows. Butan-1-ol was refluxed for 2 hours with potassium hydroxide (1%, w/v) and distilled. Light petroleum was shaken overnight with concentrated $H_2SO_4$ (10%, w/v), washed free of acid with distilled water, and distilled. Pyridine (Mallinckrodt) was refluxed with potassium hydroxide (1%, w/v) and distilled. Analytical reagent grade preparations of other solvents were used without further purification.

[2] P. Stoffyn, personal communication.

## EXPERIMENTS AND RESULTS

Methylation and partial acid hydrolysis of KS I were carried out as summarized in Diagram 1.

### *Methylation (10)*

Keratosulfate (1.05 g) was dissolved in freshly distilled formamide (8 ml) by shaking for 1 hour in a stoppered flask. Pyridine (8 ml) and acetic anhydride (4 ml) were added, and the mixture was shaken overnight. A further portion of acetic anhydride (6 ml) was added the next day, and the shaking was continued for 24 more hours. The reaction mixture was poured with stirring into cold ethanol (400 ml) containing potassium acetate, and the precipitate was collected by centrifugation. The precipitate was dissolved in water (50 ml) and reprecipitated from ethanol; this process was repeated once. The precipitate was washed twice with ethanol, dissolved in water, and lyophilized to yield 0.97 g (71%)[3] of the acetylated product.

[3] The poor yield could at least be partly explained by the failure to allow enough time for the precipitation of the acetylated keratosulfate from ethanol. When KS II was acetylated by the

TABLE I

*Gas-liquid chromatographic examination of methyl glycosides of methylated neutral sugars from KS I*

These experiments were carried out by Professor G. O. Aspinall, Department of Chemistry, University of Edinburgh.

| Compound | Retention times[a] | |
|---|---|---|
| | XE-60, 125° | NPGA, 175°[b] |
| 2,3,4-Tri-*O*-methyl-L-fucose | | 0.70 |
| 2,3,4,6-Tetra-*O*-methyl-D-galactose | 1.95 | 1.57 |
| Unknown | 2.69 | 2.32 |
| 2,4,6-Tri-*O*-methyl-D-galactose | 3.99; 3.52 | 3.30; 3.00 |
| 2,3,4-Tri-*O*-methyl-D-galactose | 6.95 | 4.85; 5.58 |
| 2,4-Di-*O*-methyl-D-galactose | 14.58; 13.40 | 10.5; 9.48 |

[a] Retention times relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

[b] NPGA, neopentyl glygol adipate.

TABLE II

*Fractionation of hydrolysate from methylated KS I*

| Fraction | Weight | $R_G$ | Probable identity of sugar |
|---|---|---|---|
| | mg | | |
| 1 | 5.8 | 0.90 | 2,3,4,6-Tetramethylgalactose and 2,3,4-trimethylfucose |
| 2 | 62.6 | 0.79 | 2,4,6-Trimethylgalactose |
| 3 | 41.7 | 0.52 | 2,4-Dimethylgalactose |
| 4 | 30.8 | 0.56 | 3,6-Dimethyl-*N*-methylglucosamine |
| | | 0.39 | 3,6-Dimethylglucosamine |
| | | 0.23 | Fraction 5 |
| 5 | 72.4 | 0.24 | 3-Methyl-*N*-methylglucosamine |
| | | 0.18 | 3-Methylglucosamine |

Acetylated keratosulfate (0.91 g) was dissolved in dimethylformamide (30 ml) and maintained in an ice bath under nitrogen with continuous stirring. Powdered sodium hydroxide (17.5 g) and dimethyl sulfate (21 ml) were added in seven equal portions over a period of 48 hours. The mixture was neutralized with dilute sulfuric acid to pH 7.0 and filtered, and the residue was repeatedly extracted with chloroform. The filtrate and chloroform extracts were evaporated under reduced pressure to yield Syrup *a*. The residue after chloroform extraction was dissolved in water and dialyzed, first against running tap water and then against distilled water. On concentration the bag contents yielded Syrup *b*, which gave a positive anthrone test, showing that a part of the partially methylated keratosulfate is insoluble in chloroform. Syrups *a* and *b* were combined and again treated with dimethyl sulfate (21 ml) and powdered sodium hydroxide (17.5 g) as described above. The reaction mixture was neutralized and dialyzed against running tap water for 48 hours, and then against two changes of stirred distilled water at 4° for 48 hours. The bag contents were filtered, concentrated to a small volume, and lyophilized, yielding 0.51 g of partially methylated keratosulfate[4] ($OCH_3$, 16.9). This product was dissolved in dimethylformamide (15 ml) and dry methanol (5 ml) and methylated by Kuhn's method (11) with BaO (6 g), $Ba(OH)_2 \cdot 8H_2O$(3 g), and methyl iodide (15 ml) to yield a yellow powder (0.50 g) ($OCH_3$, 17.6). The infrared spectra showed a peak at 3200 to 3500 $cm^{-1}$ due to hydroxyl or —NH absorption, or both. The material was finally treated with distilled dry methyl iodide and freshly prepared silver oxide (12, 13) in purified dimethylformamide. A glassy yellow solid (0.44 g, 42% yield) was obtained, which was only partially soluble in water and chloroform but was completely soluble in methanol and dimethylformamide. Calculated: $SO_4$, 13.3; $OCH_3$, 23.8. Found: $[\alpha]_D^{20}$ +4(*c*, 1.0); $SO_4$, 11.5; $OCH_3$, 22.7. The Seliwanoff test (14) for anhydro sugars on this material was practically negative.

*Hydrolysis of Methylated Polysaccharide*

The methylated product (400 mg) was dissolved in 2% methanolic hydrogen chloride (30 ml) and refluxed at 100° for 6 hours. The solution was concentrated to a syrup under reduced pressure below 35° and further dried over sodium hydroxide pellets in a desiccator. The mixture of methyl glycosides thus obtained was dissolved in N $H_2SO_4$ (45 ml) and heated at 100°. The hydrolysis was followed by determination of reducing sugar (15) and stopped after 7 hours, when the maximum reducing value had been attained. The hydrolysate was neutralized with $Ba(OH)_2$, followed by $BaCO_3$, and filtered, and the precipitate was washed with water and then methanol. The filtrates were concentrated under reduced pressure, yielding a syrup, which was extracted with dry methanol and centrifuged; the supernatant was dried to yield 0.39 g of methyl ethers.

*Methylation Results*

Preliminary paper chromatograms in Solvents A, B, and C and thin layer chromatography in Solvents *a* and *b* showed seven methyl ether components and no unmethylated or monomethylated sugars, confirming that a fairly high degree of methylation was obtained. A sample (8 mg) of the methylated monosaccharides was dried under vacuum and converted to the methyl glycosides by heating at 100° with 3% methanolic hydrogen chloride in a sealed tube for 6 hours. The product isolated after neutralization with silver carbonate was applied as a water solution on to a column of Dowex 50-$H^+$ (12 × 1.5 cm). The column was eluted with water until the washings gave a negative reaction with anthrone. The water eluate, which contained only the methyl glycosides of methylated neutral sugars, was dried and examined by gas-liquid chromatography (16). The results are summarized in Table I.

*Fractionation of Methylated Monosaccharides*

The syrup (350 mg) was lyophilized on 3 g of cellulose powder, and this powder was then packed on top of a cellulose column (51 × 2 cm). The column was successively eluted with light petroleum (b.p. 110–115°)-butan-1-ol (7:3, later 1:1) saturated with water; butan-1-ol half saturated with water; and water. Fractions of about 10 ml were collected every 20 min. Every fifth fraction was taken to dryness, examined chromatographically in Solvent B, and appropriately bulked to give five fractions (see Table II).

same procedure and the reaction mixture, after being poured into ethanol, was allowed to stand overnight in the refrigerator, a 97% yield of the product was obtained (unpublished results).

[4] This material was easily soluble in methanol, chloroform, and dimethylformamide, but gave a turbid solution in water.

*Fraction 1*—The syrup (5.8 mg) had the same mobility as 2,3,4,6-tetra-*O*-methylgalactose on paper chromatograms in Solvents A, B, and C. Demethylation (1 mg) with hydriodic acid followed by chromatography in Solvent D showed galactose and a trace of a second component with the mobility of fucose. The sugar (4 mg) was refluxed with redistilled aniline (2 mg) in absolute ethanol (1 ml) for ½ hour. The product resisted all attempts to crystallize it. The methyl ethers were recovered by treatment with Dowex 50-$H^+$. Thin layer and gas-liquid chromatography showed that the main component in this fraction was 2,3,4,6-tetra-*O*-methylgalactose.

*Fraction 2*—The sugar, $[\alpha]_D$ +82.8 (*c*, 1.20), was chromatographically indistinguishable from 2,4,6-tri-*O*-methyl-D-galactose in Solvents A, B, and C. Demethylation with hydriodic acid gave only galactose. A portion (20 mg) was refluxed for 30 min with redistilled aniline (10 mg) in absolute ethanol (2 ml) with exclusion of light. On removal of the solvent, a partially crystalline material was obtained which recrystallized twice from ethyl acetate; m.p. and mixed m.p. with authentic 2,4,6-tri-*O*-methyl-*N*-phenyl-D-galactosylamine, 195–197°. The rest of the sugar crystallized after prolonged desiccation and was recrystallized from ethyl acetate-light petroleum; m.p. 98–99°; mixed m.p. with authentic 2,4,6-tri-*O*-methyl-D-galactose, 97–100°.

*Fraction 3*—The syrup, $[\alpha]_D$ +78.0 (*c*, 0.91), was chromatographically pure and identical with 2,4,di-*O*-methyl-D-galactose in Solvents A, B, and C. The demethylated product showed only galactose on paper chromatograms run in Solvents B and D. The syrup crystallized on desiccation and was recrystallized from ethyl acetate; m.p. 94–97°. The aniline derivative was prepared as described above for Fraction 2; m.p. 210–212°; mixed m.p. with authentic 2,4-di-*O*-methyl-*N*-phenyl-D-galactosylamine, 212–214°.

*Fraction 4*—The syrup (30.8 mg) was fractionated on Whatman No. 3MM paper in Solvent B into three fractions.

*Fraction 4a*—(5.3 mg) was chromatographically indistinguishable from Fraction 5 in Solvents B and D, and the two fractions were therefore combined.

*Fraction 4b*—This fraction (14.1 mg; $R_G$ 0.39; $R_F$ in Solvent B, 0.36) gave only one spot on paper chromatography in Solvents A, B, and D. This spot gave positive reactions with aniline phthalate and ninhydrin sprays, but did not react with the Morgan-Elson spray, even after a long time. Paper electrophoresis in Buffer I at 500 volts for 2 hours gave only one spot with the same mobility as D-glucosamine. Demethylation with hydriodic acid followed by treatment with ninhydrin (17) showed mainly arabinose in a paper chromatogram run in Solvent D. Direct ninhydrin treatment of the fraction gave a sugar which had the same mobility on paper and thin layer chromatograms as the sugar obtained by ninhydrin treatment of Fraction 4c (see "Discussion"). The syrup (5 mg) was *N*-acetylated (1) to give a product which had a chromatographic mobility identical with that of authentic 2-acetamido-2-deoxy-3,6-di-*O*-methyl-D-glucose. The product gave a positive reaction with the Morgan-Elson reagent.

*Fraction 4c*—This fraction (10.4 mg; $R_G$ 0.54; $R_F$ 0.49 in Solvent B) also gave only one spot on paper chromatograms developed in Solvents A, B, and D. The spot reacted strongly with aniline phthalate, rather faintly with ninhydrin, and very slowly with the Morgan-Elson spray, showing a reddish violet color after about 30 min (18). Paper electrophoresis in Buffer I as above (Fraction 4c) gave one spot with the same mobility as D-glucosamine. Demethylation followed by ninhydrin treatment gave mainly arabinose. Direct ninhydrin treatment of this fraction gave a sugar with a mobility corresponding to that of a dimethylarabinose on paper and thin layer chromatograms. Complete *N*-acetylation (1) of Fraction 4c was difficult to attain; even after two treatments the product showed two spots on paper electrophoresis, one with the mobility of D-glucosamine and the other staying at the origin (see "Discussion").

*Fraction 5*—The syrup (72.4 mg) was fractionated on Whatman No. 3MM paper into three fractions in Solvent B.

*Fraction 5a*—This fraction (40.2 mg; $R_G$ 0.18; $R_F$ 0.17 in Solvent B) gave a single spot on paper chromatography in Solvents B and D. The spot reacted with aniline phthalate, alkaline silver nitrate, and ninhydrin sprays, but not with the Morgan-Elson spray even after a long time. Paper electrophoresis in Buffer I gave only one spot with the mobility of glucosamine. Demethylation followed by ninhydrin gave only arabinose on paper in Solvents B, D, and G. The syrup (30 mg) was *N*-acetylated. The product, $[\alpha]_D$ +28 (*c*, 0.2), had the same Morgan-Elson and $R_F$ values as an authentic sample of 2-acetamido-2-deoxy-3-*O*-methyl-D-glucose. The *N*-acetylated product was recrystallized from methanol-ether; m.p. 158°.

*Fraction 5b*—This fraction (16.2 mg) was a mixture of Fractions 5a and 5c and was not further investigated.

*Fraction 5c*—This fraction (10.3 mg; $R_G$ 0.26; $R_F$ 0.22 in Solvent B) gave only one spot in Solvents B and D and reacted strongly with aniline phthalate and alkaline silver nitrate sprays, but only faintly with ninhydrin. The sugar reacted very slowly with the Morgan-Elson spray, giving a reddish violet spot after about ½ hour. Paper electrophoresis in Buffer I gave only one spot, with the mobility of glucosamine. Demethylation followed by ninhydrin treatment gave mainly arabinose, as shown by paper chromatography in Solvents B, D, and E. Direct ninhydrin treatment of Fractions 5a and 5c gave mainly one product, presumably a monomethylarabinose, which had similar mobilities on paper and thin layer chromatography.

### *Partial Acid Hydrolysis of KS I*

Trial hydrolyses were carried out with N acetic acid and 0.5 N $H_2SO_4$ for various lengths of time. The products were examined chromatographically in Solvents E and F and electrophoretically in Buffer I at 300 volts for 1½ hours, with aniline phthalate and ninhydrin sprays. It appeared that the best yield of sulfated and nonsulfated oligosaccharides were obtained on hydrolysis with 0.5 N $H_2SO_4$ at 100° for 1 hour. Accordingly, KS I (1.0 g) in 0.5 N $H_2SO_4$ (100 ml) was hydrolyzed for 1 hour at 100°. The cooled solution was neutralized with $Ba(OH)_2$ and $BaCO_3$ and centrifuged, and the precipitate was washed twice with water. The supernatant and washings were concentrated to a small volume, some precipitate that formed was filtered, and the clear solution was dried (756 mg). This material was redissolved in water (50 ml) and dialyzed with stirring against two changes of distilled water (400 ml each) at 4° for 48 hours. The dialysates (Fraction A) and bag contents (Fraction B) were separately concentrated and lyophilized, yielding 440 mg and 280 mg, respectively.

### *Partial Hydrolysis Results*

*Fraction A*—Fraction A was fractionated by electrophoresis on Whatman No. 3MM sheets in Buffer III at 25 volts per cm for

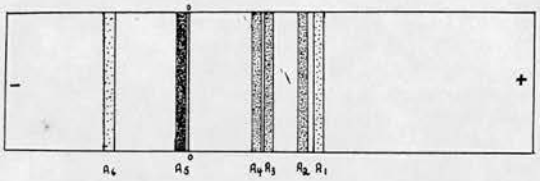


FIG. 1. Electrophoretic fractionation pattern of partial acid hydrolysate of KS I.

TABLE III

*Sulfated oligosaccharides from partial acid hydrolysis of KS I*

All fractions gave a positive aniline phthalate reaction and a negative ninhydrin reaction. After total hydrolysis (N hydrochloric acid, 16 hours, 100°), all gave only galactose and glucosamine on paper chromatograms. After sodium borohydride reduction, hexosamine was practically unchanged in the last three fractions; in the first fraction, 57.2% of hexosamine was destroyed.

| Sodium salt | Weight | $R_{Gal}$ | | Molar ratios | | | Destruction of galactose on $NaBH_4$ reduction |
|---|---|---|---|---|---|---|---|
| | | Solvent E | Solvent G | Galactose | Hexosamine | Sulfate | |
| | mg | | | | | | % |
| A2c | 7.8 | 0.34 | | 1.00 | 0.95 | 0.92 | 10.6 |
| A4a | 4.7 | 0.38 | 0.09 | 2.00 | 0.96 | 0.99 | 51.5 |
| A4b | 1.8 | 0.53 | 0.18 | 1.00 | 0.96 | 2.64 | 80.8 |
| A4c | 4.1 | 0.75 | 0.24 | 1.00 | 0.94 | 0.89 | 89.5 |

105 min into six fractions (see Fig. 1). After elution from paper with deionized distilled water, Fractions A1 through A4, which were acidic (sulfated) sugars, were neutralized with dilute $NH_4OH$ and taken to dryness. Fraction A5, which contained the neutral sugars and amino acids, and Fraction A6, containing the basic sugars, were also concentrated to dryness.

*Fraction A2*—The sample (64.6 mg) was further fractionated into three subfractions on thick filter sheets in Solvent E, and the sugars were isolated as their barium salts.

*Subfraction A2a*—This subfraction (13.2 mg) was chromatographically (Solvent E) and electrophoretically (Buffer III) identical with *N*-acetylglucosamine 6-sulfate. It gave a positive reaction with aniline phthalate and Morgan-Elson sprays, but not with ninhydrin. A sample (100 μg) on hydrolysis with N hydrochloric acid at 100° for 6 hours gave only glucosamine on paper chromatography. The infrared spectrum was identical with that of an authentic sample of *N*-acetylglucosamine 6-sulfate potassium salt. This fraction had the following analysis: *N*-acetylglucosamine (Morgan-Elson), 18.70 μM; D-glucosamine (Elson-Morgan), 18.40 μM; sulfate, 18.05 μM; and reducing sugar value (14), 18.28 μM. For periodate studies, a portion of the barium salt was passed through a small column of Dowex 50-$H^+$, eluted with deionized water, neutralized with dilute $NH_4OH$ to pH 7.0 and evaporated to dryness under reduced pressure; $[\alpha]_D$ +47.9 (*c*, 0.06, ammonium salt). A solution of the ammonium salt (0.3 mg/0.5 ml) was mixed with 0.5 ml of 0.03 M sodium metaperiodate and set aside in the dark at room temperature. Aliquots (0.1 ml) were pipetted at various intervals, and the reduction of periodate was determined according to Aspinall and Ferrier (19) (see Table IV). After 144 hours, the reaction mixture was tested for formaldehyde with chromotropic acid (20). Only insignificant amounts of formaldehyde were detected. Subfraction A2a (0.94 μM) was oxidized with 0.012 M sodium metaperiodate in 5 ml of sodium bicarbonate solution (0.04 M, pH 7.5). *N*-Acetyl-D-glucosamine (0.84 μM) was also oxidized under the same conditions. Blanks of Subfraction A2a and inactivated sodium metaperiodate were also included. Aliquots (1 ml) were withdrawn after 24 and 46 hours, and formaldehyde was estimated as before. After 46 hours, 1 mole of A2a liberated only 6% formaldehyde compared to the amount liberated by 1 mole of *N*-acetyl-D-glucosamine.

*Subfraction A2b*—The sugar (12.3 mg) had $[\alpha]_D$ +42.5 (*c*, 0.11) and a mobility of 0.90 relative to glucose 6-sulfate in Solvent E. Electrophoresis in Buffer III gave a single spot with a mobility identical with that of authentic glucose 6-sulfate. The fraction gave a positive reaction with aniline phthalate and triphenyltetrazolium salt (21) sprays. Paper chromatography in Solvents E and G of a hydrolyzed (N hydrochloric acid, 100°, 6 hours) sample showed only galactose. A portion of the fraction was converted to the ammonium salt as described for Fraction A2a; $[\alpha]_D$ +52.0 (*c*, 0.09, ammonium salt). The fraction had a degree of polymerization (22) of 0.84 and a molar ratio of sulfate to galactose of 1.06. The infrared spectrum showed a strong absorption at 1240 and 820 $cm^{-1}$. The ammonium salt (0.325 mg) was oxidized with sodium metaperiodate as described for Fraction A2a (see Table IV). A test for formaldehyde (20) at the end of 144 hours was negative. Periodate oxidation in sodium bicarbonate buffer for 46 hours liberated about 4% of the formaldehyde liberated by an equal amount of D-galactose.

*Subfraction A2c*—This subfraction (30.7 mg) was a mixture of several components as shown by chromatography in Solvents E, G, and H. One major component (7.8 mg; mobility relative to glucose, 0.29, in Solvent H) was isolated by preparative chromatography in Solvent G. This component was chromatographically (Solvents E, G, and H) and electrophoretically (Buffer III) homogeneous. Complete hydrolysis with N hydrochloric acid for 6 hours at 100° gave galactose and glucosamine. Mild hydrolysis (0.5 N sulfuric acid, 1 hour, 100°) followed by chromatography and electrophoresis showed, in addition to galactose and glucosamine, some unhydrolyzed material, an unidentified sugar, and a small amount of galactose 6-sulfate. A portion was reduced with potassium borohydride, hydrolyzed (0.5 N hydrochloric acid, 9 hours, 100°), and examined by chromatography. Galactose, glucosamine, glucosaminitol, and a trace of galactitol were detected. The analysis of the compound before and after borohydride reduction is given in Table III.

*Fraction A4*—This fraction (105 mg) was fractionated on a Bio-Gel P2 column (95 × 3 cm). The fractions were screened for anthrone, and then every fifth fraction was taken to dryness and examined chromatographically in Solvents E and G. By combining appropriate tubes, nine fractions were obtained. The three major fractions were purified further on paper to give chromatographically and electrophoretically homogeneous Fractions A4a, A4b, and A4c, which were isolated as their sodium salts. Some of the analyses of these fractions are summarized in Table III.

*Fraction A4a*—Yeast β-galactosidase, which almost completely digested lactose[5] in 15 hours, did not have any effect on this

[5] The main products were galactose and glucose; however, some unchanged lactose and two slower moving components, presumably trisaccharides, were present. This suggests the occurrence of a transglycosidase in yeast β-galactosidase.

fraction. Mild acid hydrolysis (0.25 N sulfuric acid, 1 hour, 100°) followed by paper chromatography showed *N*-acetyllactosamine, galactose, and *N*-acetylglucosamine, among other products.

## DISCUSSION

In the earlier investigation (1), keratosulfate was methylated by four successive treatments with Haworth's reagents. A product with a methoxyl content of about 80% of the theoretical value was obtained, which, on account of its insolubility in organic solvents, could not be further methylated with the conventional Purdie reagent (12). In general, the small amounts of keratosulfate available limit the choice of methylation techniques. Methylation in nonaqueous solvents appears in general to be more efficient than the classical Haworth technique. The latter, which is carried out in aqueous media, even though mild (e.g. it is not known to cause *N*-methylation), requires repeated treatments, sometimes up to six or seven times, to approach full methylation. This method was not applied here, since poor yields could be expected as a result of the losses from the repeated treatments. In the present investigation, the procedure of Bouveng, Kiessling, Lindberg, and McKay (10) was used. Keratosulfate was acetylated in order to obtain products soluble in organic solvents in which they were methylated. This procedure, in addition to giving a high degree of methylation, keeps the alkaline degradation to a minimum. The methylation according to Hakamori (23), even though now widely used, was not utilized here because of the strongly alkaline nature of the methylsulfinyl carbanion. This strong alkalinity could cause extensive degradation, especially in the case of a polysaccharide such as keratosulfate, which has 1 → 3-linked sugars.

The acetylation procedure was also expected to *N*-acetylate any amino groups present in the polymer. It is known that free amino groups are *N*-methylated by dimethyl sulfate and sodium hydroxide (24), whereas *N*-acetylhexosamine is not affected by these reagents (25). Our methylation procedure caused at least partial *N*-methylation of *N*-acetylhexosamine, which most probably occurred during the treatment with Kuhn's reagent. This was also suspected by Venkata Rao, Buchanan, and Baddiley (26), who used Kuhn's procedure for methylation. The method of Hakamori causes complete *N*-methylation of *N*-acetylhexosamine.[6]

The acetylated keratosulfate was first methylated in dimethylformamide with the use of dimethyl sulfate and sodium hydroxide. It was found that a part of the methylated keratosulfate remained insoluble in chloroform. This is not unexpected, because of the presence of sulfate groups. Hence, after the second treatment, the product was isolated by dialysis. This was found desirable, even at the expense of the yield, on account of loss of degradation products of low molecular weight. Dialysis also removed dimethylformamide and the small amounts of impurities which are contained even in the purified solvent. Dimethylformamide otherwise is difficult to remove even by repeated evaporation with solvents such as 1-propanol, with which it is known to form a low boiling azeotrope. The nondialyzable product had 71% of the theoretical methoxyl content. After one treatment by Kuhn's procedure, the value increased by 2%. The infrared spectrum at 3200 to 3600 $cm^{-1}$ showed only a small peak compared to that of the unmethylated polysaccharide. It is not known whether this peak is due to hydroxyl or —N—H absorption. Hydrolysis and chromatographic examination of a small sample at this stage showed traces of free (unmethylated) galactose and glucosamine. The methoxyl value after treatment with methyl iodide and silver oxide was 86.5% (w/w) of the theoretical value, the latter on the basis of a monosulfated *N*-acetyllactosamine repeating unit. This product gave a cloudy solution in water and had $[\alpha]_D$ +4.0 (*c*, 1.0). The high peptide content (10.3%) of the starting material and the balance of sugar, sulfate, and peptide analysis, which do not add up to 100%, probably explain the methoxyl value. No further treatments were attempted since methylation appeared complete. Some desulfation did occur during methylation, as indicated by the decreased sulfate value (86.5% of theory). This is not surprising in view of the alkali lability of sulfate esters. The Seliwanoff test for anhydro sugar, such as 3,6-anhydrogalactose, was negligible. If, as is concluded below, all galactose residues are 3-linked, then no 3,6-anhydrogalactose could be formed, except from nonreducing 6-sulfated galactose end groups. On the other hand, *N*-acetylglucosamine is mainly 4-linked and could, under alkaline conditions, form 3,6-anhydro-*N*-acetylglucosamine. It is not known whether this sugar gives a positive Seliwanoff test.

The methylated keratosulfate was first methanolyzed, and the water-soluble mixture of methyl glycosides was further hydrolyzed with N sulfuric acid until the reducing sugar value did not increase. A small portion of the hydrolysates was reconverted to methyl glycosides, and the methyl glycosides of the methylated neutral sugars obtained free of amino sugars (on Dowex 50-$H^+$) were examined by gas-liquid chromatography (16). The results indicated that the two major components were 2,4,6-tri-*O*-methylgalactose and 2,4-di-*O*-methyl-D-galactose. In addition, small amounts of two unknowns and trace amounts of 2,3,4,6-tetra-*O*-methylgalactose and 2,3,4-tri-*O*-methylfucose were present. The two major sugars were characterized by preparation of crystalline derivatives. Fraction 4b was identical in its properties with 3,6-di-*O*-methylglucosamine. Fraction 4c, which had a slightly faster mobility on paper than 4b, reacted rather differently with ninhydrin. In the Morgan-Elson reaction, this fraction reacted slowly and gave a reddish violet color in about ½ hour. As a model compound, *N*-methyl-L-glucosamine was prepared by hydrolysis (2 N hydrochloric acid, 4 hours, 100°) of pentaacetyl-*N*-methyl-L-glucosamine. This sugar (mobility relative to glucosamine of 1.37 in Solvent G) reacted with aniline phthalate spray and rather faintly with ninhydrin. Whereas glucosamine does not give a color in the Morgan-Elson reaction, *N*-methylglucosamine gave a reddish violet color which developed rather slowly (30 to 60 min). *N*-Acetylglucosamine, however, develops a violet color in 5 min. On paper electrophoresis in Buffer III, *N*-methylglucosamine had almost the same mobility as glucosamine, whereas *N*-acetylglucosamine stayed at the origin. On ninhydrin oxidation (17), *N*-methylglucosamine was partly converted to arabinose (about 60% as judged by paper chromatography). The NMR spectra of pentaacetyl-β-D-glucosamine and pentaacetyl-*N*-methyl-L-glucosamine in $CDCl_3$ solutions were recorded with a Varian A-60A NMR spectrometer.[7] A single sharp absorption (area, 3 hydrogens) is observed; $\tau$ = 7.09 (tetramethylsilane was used

[6] S. I. Hakamori and R. W. Jeanloz, personal communication.

[7] We are grateful to Dr. I. J. Borowitz for the use of the NMR spectrometer, purchased through Grant A1-07455-01 from the National Institutes of Health, and to Dr. Karl Untch for running the spectra.

TABLE IV

*Reduction of periodate by Fractions A2a and A2b*

| Fraction | Reduction of periodate in | | | | | |
|---|---|---|---|---|---|---|
| | 1 hr | 4 hrs | 24 hrs | 48 hrs | 96 hrs | 144 hrs |
| | *moles/mole of fraction* | | | | | |
| A2a | 0.85 | 1.15 | 3.85 | 4.24 | 5.18 | 5.26 |
| A2b | 2.94 | 3.53 | 3.64 | 3.83 | 3.90 | 3.92 |

TABLE V

*Analysis of nondialyzable Fraction B after partial hydrolysis of KS I*

| Material | Anthrone | Hexosamine | Sulfate | Protein | Orcinol | Carbazole |
|---|---|---|---|---|---|---|
| | % | % | % | % | % | % |
| KS I | 35.3 | 26.1 | 14.9 | 10.3 | 8.0 | 1.8 |
| Fraction B | 26.9 | 27.4 | 7.1 | 15.8 | 8.4 | 1.4 |

as internal standard) in the case of the *N*-methyl derivative. This value of the chemical shift for the *N*-methyl group of the latter compound is distinctly different from those due to the *O*- and *N*-acetylmethyl groups ($\tau$ = 7.80 to 8.10) (27). Thus, this method appears to be extremely useful and direct for the detection of *N*-methylated hexosamines.

It thus appeared that Fraction 4c is an *N*-methylglucosamine derivative. Ninhydrin oxidation of Fractions 4b and 4c gave the same dimethylarabinose derivative. This strongly indicates that Fraction 4c is 3,6-di-*O*-methyl-*N*-methyl-D-glucosamine. Even after two *N*-acetylations, this fraction gave two spots on paper electrophoresis: one with the mobility of D-glucosamine (presumably 3,6-di-*O*-methyl-*N*-methyl-D-glucosamine), and one at the origin (3,6-di-*O*-methyl-*N*-methyl-*N*-acetyl-D-glucosamine). This indicated that the conditions used for complete *N*-acetylation of D-glucosamine are not sufficient to cause complete *N*-acetylation of *N*-methylglucosamine. Fraction 5a was characterized as 3-*O*-methyl-D-glucosamine by preparation of crystalline 3-*O*-methyl-*N*-acetyl-D-glucosamine. A set of reactions similar to those described above suggested that Fraction 5c is 3-*O*-methyl-*N*-methyl-D-glucosamine.

Partial hydrolysis of the polysaccharide following electrophoretic separation of the dialyzable fragments gave the pattern shown in Fig. 1. Fraction A5 consisted of the expected neutral sugars (galactose, *N*-acetylglucosamine, and four oligosaccharides) together with amino acids and peptides. Glycopeptides were present in this fraction. Fraction A6 was mainly glucosamine, with at least two basic oligosaccharides which gave galactose and glucosamine on hydrolysis. Fractions A1 to A4 consisted of the acidic sugars, the main fractions being A2 (64.6 mg) and A4 (105.0 mg). Fraction A2, after further fractionation on filter paper sheets, gave three compounds, A2a, A2b, and A2c. A2a and A2b were characterized as *N*-acetylglucosamine 6-sulfate and galactose 6-sulfate, respectively. Characterization was made by chromatographic and electrophoretic mobility, hydrolysis products, color reactions, infrared spectra, and periodate oxidation (Table IV). The fast reduction of 2.94 moles, followed by a slow reduction of 1 mole of periodate and liberation of only a negligible amount of formaldehyde, is in accordance with the expected values for galactose 6-sulfate (28). In the case of *N*-acetylglucosamine 6-sulfate, however, even though the formaldehyde liberated was negligible as expected, the reduction of periodate did not agree with the theory. The reduction kept steadily increasing and even after 96 hours did not stop completely. Jeanloz and Forchielli (29) showed that the *N*-acetyl group does not prevent the cleavage of adjacent —C(OH)—C($NH_2$)— and that *N*-acetylglucosamine consumes 5 moles of periodate under ideal conditions. Fraction A2c was a complex mixture. The major component (mobility relative to glucose, 0.29, in Solvent H) was isolated after fractionation on Whatman No. 3MM paper. The ratio of galactose to hexosamine to sulfate was approximately 1:1:1. On sodium borohydride reduction, 57.2% hexosamine and 10.6% galactose were destroyed. Thus it appeared that Fraction A2c is a tetrasaccharide with a hexosamine at the reducing end. The tetrasaccharide would have two sulfate groups, at least one on galactose, since some galactose 6-sulfate was detected in the partial hydrolysate of A2c. From Fraction A4, three sulfated oligosaccharides (A4a, A4b, and A4c) were obtained in pure form. Fraction A4a is a sulfated trisaccharide with galactose at the reducing end. The ratio of galactose to hexosamine to sulfate was approximately 2:1:1. Roughly half the galactose was destroyed, whereas hexosamine was unchanged after sodium borohydride reduction. Two structures, namely Gal–GlcNAc–Gal and GlcNAc–Gal–Gal, are possible for this trisaccharide. Since *N*-acetyllactosamine was detectable among the partial acid hydrolysis products of A4a, the probable structure is Gal–GlcNAc–Gal. Yeast $\beta$-galactosidase was without effect on this trisaccharide. This may indicate that galactose at the nonreducing end is sulfated. Sulfation of glycosyl group in general causes resistance to glycosidases (30). Not enough material was available for a desulfation followed by $\beta$-galactosidase digestion. Fraction A4b is *N*-acetyl-D-glucosaminyl-D-galactose. The ratio of galactose to hexosamine was 1:1. Sodium borohydride reduction caused destruction of 81% of galactose, with no change in hexosamine. The high sulfate content of A4b could at least be partly explained by a disulfated disaccharide fraction. Fraction A4c appears from the analysis to be *N*-acetylglucosaminyl-D-galactose with a sulfate group substituted on position 6 of one of the sugar residues.

The methylation and partial hydrolysis results are in general agreement with the structure proposed previously (1, 31) for KS 1, namely, a chain made up of the repeating unit (1 → 3)-$\beta$-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-$\beta$-D-glucopyranose. In the earlier work it was suspected that all the sulfate was substituted at position 6 of *N*-acetylglucosamine. This conclusion was arrived at on the assumption that the dimethylgalactose from the methylated native polymer was due to undermethylation. In this reinvestigation undermethylation seemed highly improbable. Thus, approximately 40% of the galactose residues and 74% of the *N*-acetylglucosamine groups are sulfated in position 6. The exact distribution and sequence of the sulfate groups cannot be evaluated from these studies. The substitution may be random or systematic and consequently coded. In any event, the isolation of disulfated *N*-acetyllactosamine from a polymer with a sulfate to hexosamine ratio no greater than 1 requires the presence in the polymer of regions of nonsulfation. These could be present as nonsulfated side chains in the proteinpolysaccharide or as a nonsulfated repeating unit in the same polysaccharide side chain. A trace amount of 2,3,4-trimethylgalactose detected only by gas-liquid chromatography presumably arises from 6-substituted terminal galactose

residues. The presence of 2,3,4,6-tetramethylgalactose and 2,3,4-trimethylfucose indicates nonreducing galactose and fucose residues in the molecule. These residues could arise either from different chains where they are the terminating end groups, or, more likely, from the same chain where one (presumably galactose) is the chain-terminating sugar and the other (fucose) is a single branch unit. In either case, fucose could be attached to position 6 of either galactose or *N*-acetylglucosamine. Final proof of the structure is expected from isolation of fucosyl-oligosaccharides from enzymatic digests. KS I, in any event, seems to have little branching as compared to its skeletal counterpart.[8] The nondialyzable fragment from partial hydrolysis (Fraction B) of the polysaccharide had a galactose to hexosamine ratio of 1.00:1.03 (Table V) as compared to 1.00:0.75 in the original polysaccharide. The excess of galactose over hexosamine, which amounts to approximately 25%, and its preferential loss by mild hydrolysis and methanolysis during catalytic desulfation[8] are difficult to explain. The excess of galactosyl groups could be present as blocks on the nonreducing ends of the polysaccharide chains or as branches. If present as branches, the galactosyl groups would have to be substituted in position 3, possibly by sulfate. Unsubstituted galactosyl end groups would have resulted in a larger amount of tetramethylgalactose. On the other hand, the infrared spectrum gave no evidence for the presence of galactose 3-sulfate in the polysaccharide. From Table V it is obvious that 55% of the protein in the original polymer became dialyzable as a result of the very mild acid hydrolysis. This was also evident by the detection of amino acids or peptides, or both, in Fraction A5 of the dialyzable fragments.

*Acknowledgments*—We are indebted to Miss Diana Yung for skillful assistance; to Professor G. O. Aspinall for gas-liquid chromatography and its interpretation; and to him, Professor R. Kuhn, Dr. K. O. Lloyd, Dr. B. A. Lewis, and Merck and Company, Inc., for authentic samples.

[8] V. P. Bhavanandan and K. Meyer, manuscript in preparation.

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Reprinted from the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
Vol. 58, No. 4, pp. 1655–1659. October, 1967.

# *THE KERATOSULFATE-LIKE MUCOPOLYSACCHARIDE OF CHICK ALLANTOIC FLUID**

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*Communicated August 4, 1967*

A family of sulfated uronic acid-free mucopolysaccharides which was named keratosulfate has been isolated after proteolytic digestion from cornea, nucleus pulposus, and cartilage of mammals, birds, and fishes. These mucopolysaccharides were composed of D-galactose and hexosamine in a ratio of somewhat greater than 1, contained variable amounts of sialic acid and fucose, and had a ratio of sulfate to hexosamine of 1 or greater. The mucopolysaccharide fractions were covalently linked to proteins or peptides and in fact were highly resistant to proteolytic digestion. Thus they could either be regarded as sulfated mucopolysaccharides or sulfated glycoproteins resembling blood group substances. The latter, however, have never been found to occur as sulfate esters.

In the last few years a group of Norwegian authors reported the isolation of a uronic acid and sialic acid-free sulfated mucopolysaccharide[1] from the allantoic fluid of embryonated eggs, either normal or infected with influenza virus.[1–3] This mucopolysaccharide, termed "host factor" (HF), was obtained without proteolytic digestion, and proved to be antigenic in man and in rabbit.[1–4] Attempts to produce antibody to keratosulfates have thus far failed.[5] In fact, a substance immunologically identical with HF was shown to be present as a component of the influenza virus envelope.[6] Since the chemical structure of keratosulfate both of cornea and of cartilage has been determined in our laboratory,[8, 11, 12] it seemed of interest to study the substances present in allantoic fluid and if possible to determine why, in contrast to keratosulfates, they are antigenic. This report deals with the isolation and chemical properties of the chick allantoic fluid saccharide (CAFS). The fractions isolated, while immunologically similar or identical with the Norwegian material, showed chemical differences, the most important being a high sialic acid content.

*Materials and Methods.*—The mucopolysaccharide of chick allantoic fluid infected with influenza virus (Japan/170/62$A_2$) was isolated by methods analogous to those used in this laboratory for the isolation of keratosulfate. After removal of the virus, the supernatant fluid was precipitated with acetone. (We thank Eli Lilly and Co., Indianapolis, for the generous gift of this material.) Aliquots of the dry powder, corresponding to 16 liters of fluid, were digested with activated papain at 65° for two days, followed after neutralization by repeated digestion with Pronase, dialysis, removal of residual protein, and fractionation by free-flow electrophoresis and by Biogel. The final yield of material was 12.6 mg per liter, i.e., about six to seven times larger than that reported by Haukenes *et al.*[1]

A second batch was purified in a similar fashion. After Biogel P2 fractionation it was further fractionated on DEAE Sephadex and eluted with a linear gradient of 0.3–1.5 *M* NaCl. The main fraction was eluted in a fairly sharp peak from 0.55 to

0.85 *M* NaCl. A small amount (~8%) of a sialic acid and sulfate-free glycoprotein appeared at the start of the gradient.

*Results.*—Tables 1 and 2 give analytical values for the two samples of CAFS. For comparison, values are also given for typical fractions of cornea keratosulfate (KSI) and human cartilage keratosulfate (KSII).

Compared to KSI and KSII, CAFS contained a high proportion of galactosamine, a considerably lower sulfate and a higher sialic acid content. Noteworthy also are the differences in the amino acids between CAFS and the keratosulfates. In the case of CAFS, serine and threonine constituted about 50 per cent of the amino acids and proline about 16 per cent.

Electrophoresis on Sepraphore at pH 5.0 showed one sharp spot staining both with Alcian Blue and with Procion Brilliant Blue (for protein).[7] Electrophoresis at pH 1.8 (4% formic acid) showed an additional smaller spot of lower mobility. A sample obtained from Norway gave two spots at pH 1.8 with mobilities identical to those of our preparations; in addition there was an immobile fraction at the origin.

Our preparations were nondialyzable and were excluded by Biogel P2 and P4.

TABLE 1

COMPOSITION OF TWO PREPARATIONS OF CAFS, KSI, AND KSII

| | Chicken Allantoic Fluid Saccharide Preparation | | Keratosulfate | |
|---|---|---|---|---|
| Analysis | A(%) | B(%) | KSI(%) | KSII(%) |
| Anthrone | 26.0 | 28.1 | 35.3 | 28.7 |
| Hexosamine* | | | | |
| GlcN | 12.0 | 11.9 | 25.1 | 21.4 |
| GalN | 8.6 | 8.8 | 1.1 | 2.7 |
| Total | 20.6 | 20.7 | 26.2 | 24.1 |
| Sulfate | 6.9 | 6.0 | 17.7 | 18.9 |
| Sialic acid | 13.0 | 12.7 | Trace | 3.4 |
| Fucose | 1.0 | N.d.† | Trace | 2.7 |
| Mannose | — | N.d. | + | + |
| Protein (Lowry) | 6.0 | 4.1 | 2.5 | 6.6 |
| Protein‡ | 12.4 | 11.1 | 2.6 | 6.0 |
| Carbazole§ | 3.7 | N.d. | 3.4 | 2.4 |
| $[\alpha]_D$ (Water) | −6.8 | −3.2 | +0.5 | −7.2 |
| $SO_4$/GalN(molar) | 1.06 | 0.94 | 1.31 | 1.65 |
| GalN/GlcN(molar) | 2.17 | 2.36 | 1.40 | 1.34 |

* Not corrected for destruction during hydrolysis.
† Not determined.
‡ By amino acid analyzer.
§ The color has a different absorption than uronic acid.

TABLE 2

AMINO ACIDS OF TWO PREPARATIONS OF CAFS, KSI, AND KSII

| | Chicken Allantoic Fluid Saccharide Preparation | | Keratosulfate | |
|---|---|---|---|---|
| Amino acids | A(%) | B(%) | KSI(%) | KSII(%) |
| Aspartic acid | 2.68 | 0.78 | 39.93 | 13.34 |
| Threonine | 23.65 | 26.96 | 6.55 | 13.00 |
| Serine | 25.38 | 27.91 | 8.21 | 10.72 |
| Glutamic acid | 8.05 | 4.57 | 13.86 | 23.35 |
| Proline | 15.56 | 17.58 | 0.00 | 14.11 |
| Glycine | 1.51 | 0.73 | 9.64 | 8.80 |
| Alanine | 6.39 | 5.91 | 5.65 | 5.73 |
| Valine | 4.21 | 3.84 | 12.05 | 4.14 |
| Methionine | 1.01 | 0.90 | 0.00 | 0.28 |
| Isoleucine | 2.80 | 2.63 | 2.18 | 2.07 |
| Leucine | 6.67 | 6.59 | 1.13 | 1.37 |
| Tyrosine | 0.52 | 0.34 | 0.00 | 0.86 |
| Phenylalanine | 1.51 | 1.26 | 0.75 | 1.88 |

On Biogel P6 they were partly excluded and partly held, showing polydispersity. Analysis of the excluded and retarded fractions did not show significant differences.

On treatment with 0.5 *N* NaOH at room temperature for 48 hours, 242 $\mu$M of serine and 205 $\mu$M of threonine were selectively destroyed concomitant with 92 $\mu$M of glucosamine (or 13.3%) and 335 $\mu$M of galactosamine (65.9%). Thus the sum of serine and threonine destroyed (447 $\mu$M) was equivalent to the total hexosamines destroyed (427 $\mu$M).

In another experiment 30 per cent of glucosamine was destroyed when treated with alkali and borohydride. In this experiment, the galactosamine value could not be calculated since reduced hexosamines coincide with the galactosamine peak in the amino acid analysis.

Electrophoresis, at pH 5.0, of the alkali-treated preparation showed two spots which stained with Alcian Blue. Alkali treatment in the presence of borohydride followed by fractionation on Biogel P6 showed markedly increased polydispersity of the carbohydrate fraction. The main carbohydrate was eluted just prior to the salt peak and was free of peptide, whereas KSII after similar treatment retained its peptide chains. The approximate molecular size of this fraction corresponded to that of a hexasaccharide. A reduced chromogen (peak 3 in the amino acid analyzer) after acid hydrolysis was absent in contrast to the pattern with KSII.[8] The protein-free fraction, on mild acid hydrolysis, failed to show the unsaturated component demonstrated by Lloyd *et al.* in blood group substances.[9] As with KSII, an increase of alanine and the appearance of $\alpha$-aminobutyric acid were demonstrated after treatment with alkali and borohydride.

The only hexose detected after acid hydrolysis was galactose. Sialic acid by the Svennerholm method was 13.0 per cent and by the Warren method, 11.8 per cent. After digestion with sialidase (*Cl. perfringens*) and dialysis, sialic acid in the dialysate determined by the Warren method was 14.0 per cent. The infrared spectra of our samples were very similar to those of keratosulfate. However, the spectrum of our preparation of CAFS has an additional peak at 1400 $cm^{-1}$ which was not present in the Norwegian sample of HF. The latter preparation showed two additional peaks at 920 and 850 $cm^{-1}$, both absent in our preparations and KS. The above two peaks are typical for axial secondary sulfate groups (galactosamine or galactose-4-sulfate).[10] After acid hydrolysis with 0.5 *N* $H_2SO_4$,[11] paper chromatography and electrophoresis revealed a main spot for N-acetyl glucosamine-6-sufate and a minor spot of galactose-6-sulfate similar to those obtained with KSI and KSII. No evidence was obtained for sulfated galactosamine.

At different stages of purification, our materials were compared immunologically with purified HF obtained from Norway. In double-diffusion analysis (Ouchterlony) with antisera to HF, our samples showed two components which gave reaction of identity with HF; their reactivity in this system was not significantly altered by prior enzymatic removal of sialic acid. The same samples, however, showed only 5–10 per cent of the capacity of HF to block inhibition, by anti-HF, of viral hemagglutination.[3, 4] In quantitative precipitin analysis, CAFS exhibited approximately 20–30 per cent of the potency of purified HF in precipitating anti-HF. The sole exception to the foregoing was the protein-free oligosaccharide after treatment with alkaline borohydride. This material, even in relatively high concentration in

double-diffusion analysis, gave but one line identical with one component of HF, and was entirely inactive in the hemagglutination-inhibition-blocking test.

*Discussion.*—CAFS, although similar, is clearly distinct from the keratosulfates. Like keratosulfate it consists of a peptide backbone to which multiple short carbohydrate chains are attached by O-glycosidic linkages to serine and threonine. As in the case of KSII,[8] the sugar linking the carbohydrate chains to the peptide appears to be mainly a nonsulfated N-acetyl galactosamine. In contrast to KSII the absence of reduced chromogen on borohydride treatment of CAFS indicates the absence of 3-substitution of the galactosamine in the latter. The separation of at least the major portion of the carbohydrate covalently linked to peptide also contrasts with KSII. The larger proportion of galactosamine in CAFS as compared to KSII (Table 1) and its selective destruction during alkaline treatment would indicate that the carbohydrate chains are much shorter than those of KSII. The carbohydrate chains of both CAFS and KS consist of galactose and N-acetyl glucosamine and contain sialic acid and some methylpentose as nonreducing end groups. It is remarkable that the sulfate content of CAFS is only 6–7 per cent, as opposed to the 17–18 per cent of keratosulfate. Partial hydrolysis and infrared spectra data indicate that the sulfate ester is mainly N-acetyl glucosamine-6-sulfate. In fact, the molecular ratio of sulfate to glucosamine is close to 1.00. Since in addition to N-acetyl glucosamine-6-sulfate, a small amount of galactose-6-sulfate was detected after acid hydrolysis, the above value indicates that almost all glucosamine groups are monosulfated while galactosamine appears not to be sulfated. In contrast to KSI and KSII, CAFS is antigenically active.

The mucopolysaccharide is nondialyzable and by Biogel filtration has an apparent molecular weight greater than 4,000. The fraction isolated in this laboratory is similar but not identical with the fraction (HF) described by the Norwegian authors. The main differences lie in the yields obtained from allantoic fluid, the sialic acid content, and immunologic reactivity. Since our yield was six to seven times higher than that reported by the Norwegian authors, it seems possible that they isolated a minor fraction of the total having a lower sialic acid content and a higher immunological activity. The fractionation procedures used in the present study, including free flow electrophoresis, ion exchange, gel filtration, and electrophoresis on cellulose acetate at acid and nearly neutral pH, excluded the presence in our preparation of a nonsulfated sialic acid containing glycoprotein. The antigenicity and higher immunologic reactivity of the Norwegian material may perhaps be ascribed in part to its higher molecular weight, or to the presence of more complete peptide chains than remained in our preparations after exhaustive digestion with proteolytic enzymes.

It seems most significant in this connection, however, that the protein-free oligosaccharide fraction was still immunologically active, although only at a concentration approximately 20 times that at which other CAFS fractions reacted specifically with anti-HF. While the native protein polysaccharides of cartilage are antigenic, this property is abolished after proteolysis.

Chemically, CAFS is certainly very similar to keratosulfates. A further keratosulfate-like mucopolysaccharide, obtained from the organ of Lorenzini, was recently described and named lorenzan sulfate.[13] The latter, like KSII, did not react immunologically and probably is more closely related chemically to KSII than to

CAFS. CAFS as well as lorenzan sulfate presumably are epithelial products in contrast to the mesodermal origin of KSI and II. The former may represent a class of compounds widely distributed in epithelial mucins and cellular membranes.

We wish to express our appreciation to Dr. A. Harboe, Oslo, Norway, for a sample of his preparation of CAFS and anti-CAFS sera and to Dr. J. Doyle, Isle of Cumbrae, Scotland, for a sample of lorenzan sulfate.

Abbreviations used: CAFS, chick allantoic fluid saccharide; KSI, corneal keratosulfate; KSII, human cartilage keratosulfate; GlcN, glucosamine; GalN, galactosamine.

* This work was supported in part by grants AM-00570 and AI-03168 from the National Institutes of Health, U.S. Public Health Service.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 243, No. 5, Issue of March 10, pp. 1052-1059, 1968
*Printed in U.S.A.*

# Studies on Keratosulfates

## METHYLATION, DESULFATION, AND ACID HYDROLYSIS STUDIES ON OLD HUMAN RIB CARTILAGE KERATOSULFATE*

(Received for publication, October 11, 1967)

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### SUMMARY

**The structure of the carbohydrate moiety of old human rib cartilage keratosulfate (KS II) was investigated by methylation of the mucopolysaccharide and its desulfated analogue. The methods used were identical with those used previously for cornea keratosulfate. The methyl ethers were characterized either as crystalline derivatives or by paper, thin layer, or gas-liquid chromatography. The data are interpreted as a repeating unit of *N*-acetyllactosamine polymerized via 1 → 3-linkages to the galactose. Position 6 of *N*-acetylglucosamine or D-galactose, or both, is esterified with sulfate. The polysaccharide appears to be more highly branched than that from cornea. Furthermore, D-mannose was isolated from KS II and characterized. Mannose was also found in corneal and other keratosulfates. Catalytic desulfation in methanol yielded two high molecular weight desulfated products, one insoluble in aqueous solvents but soluble in formamide, and the other soluble in water. In addition to sialic acid and L-fucose, some D-galactose was released by methanolysis during the desulfation.**

Keratosulfate is a sulfated mucopolysaccharide composed of approximately equimolar quantities of D-galactose and *N*-acetyl-D-glucosamine and variable amounts of sulfate, L-fucose, sialic acid, and *N*-actyl-D-galactosamine. It has been isolated from cornea (1), cartilage (bovine, human, and shark) (2–4), and nucleus pulposus (5). The keratosulfates of bovine cornea and of human rib cartilage are known to differ mainly in their linkage between carbohydrate and peptide (6). The structure of the carbohydrate portion of KS I[1] was shown by Hirano, Hoffman, and Meyer (7) to be a linear molecule made up of the repeating unit (1 → 3)-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranosyl 6-sulfate. This structure was further modified by us after reinvestigation of the methylation and on the basis of studies of partial acid hydrolysis (8, 9).

KS II has been assumed to have basically the same carbohydrate structure as KS I. The studies presented in this paper support this assumption. The basic structure appears to be similar for both polysaccharides, but differences were noticed mainly in the average length of the chains and in the occurrence of a greater degree of branching in KS II. In the course of the studies on methylation of the native and desulfated polysaccharides, it was discovered that this kerotosulfate preparation contained a sugar with the chromatographic mobility of mannose. This sugar was isolated after total acid hydrolysis of KS II and characterized as D-mannose. Keratosulfates of different origin were tested for mannose, and all of them contained small amounts, roughly 1 to 2%, of this sugar. From the total acid hydrolysate, a disaccharide tentatively identified as glucosaminyl-(1 → 3)-galactose was also isolated. In addition, the catalytic removal of sulfate ester groups of keratosulfate was studied. Desulfation was carried out in such a way as to obtain a maximum yield. However, surprisingly, more than half of the desulfated product became water-insoluble.

### EXPERIMENTAL PROCEDURE

*Materials*—Rib cartilage of people over 60 years old was collected, and the keratosulfate was prepared by the procedure used in this laboratory (6). The fractions, which still had a fair amount of chondroitin sulfate and about 11% protein, were digested with testicular hyaluronidase, followed by Pronase, and fractionated with ethanol. The fraction having the following

* This work was supported by United States Public Health Service Grants AM 00570 and HD 02646. A preliminary report on this work was presented at the Fifty-first Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, April 1967, *Fed. Proc.*, **26**, 150 (1967).

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[1] The abbreviations used are: KS I, bovine cornea keratosulfate; KS II, old human rib cartilage keratosulfate. MN, methylated neutral sugars; MH, methylated hexosamines; MDN, methylated, desulfated neutral sugars; MDH, methylated, desulfated hexosamines; DKS, desulfated keratosulfate; $R_T$, retention time relative to methyl tetramethyl-α-D-glucoside.

analysis was used for the present studies: hexose (anthrone method), 31.4%; hexosamine, 28.6%; uronic acid (carbazole method), 2.1%; sulfate, 18.7%; protein (Lowry), 6.0%; methyl pentose, 2.5%; $[\alpha]_D^{23}$ −7.2°. About 11% of the hexosamine was galactosamine, and the rest was glucosamine, as determined with the amino acid analyzer.

*Sugars*—We gratefully acknowledge the following gifts of samples: 2,3,4-tri-*O*-methyl-*N*-phenyl-L-fucosylamine, 2,4,6-tri-*O*-methyl-D-galactose, and 2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose, from the late Professor R. Kuhn; 2,3,4,6-tetra-*O*-methyl- and 2,4,6-tri-*O*-methyl-*N*-phenyl-D-galactosylamine and 2,3,4-tri- and 2,3,6-tri-*O*-methylgalactose, from Professor G. O. Aspinall; 2,3,4,6-tetra-*O*-methyl-D-galactose, from Dr. K. O. Lloyd; 2,4-di-*O*-methyl-3,6-anhydro-D-galactose, from Dr. B. A. Lewis; and pentaacetyl-*N*-methyl-L-glucosamine, from Merck and Company.

*Analytical Methods*—These were the same as cited previously (9).

*Chromatography*—Paper chromatograms were developed by the descending technique on Whatman No. 1 paper. Whatman No. 3MM paper used for quantitative separation was washed with 0.01 N HCl, followed by distilled water, and finally with the solvent used in the separation. The following solvent systems (v/v) were used: A, butan-1-ol-ethanol-water (10:3:5); B, butan-1-ol-ethanol-water (4:1:5, upper layer); C, benzene-ethanol-water (169:47:15, upper layer); D, ethyl acetate-pyridine-water (10:4:3); E, ethyl acetate-acetic acid-water (6:3:2); F, ethyl acetate-pyridine-water (8:2:1); G, butan-1-ol-pyridine-water (5:3:2); H, butan-1-ol-acetic acid-ethanol-water (10:3:1:7); I, ethyl acetate-pyridine-acetic acid-water (5:5:1:3). Sugars were detected with aniline-phthalate spray or with alkaline silver nitrate. Ninhydrin and Elson-Morgan sprays were used for hexosamine, and Morgan-Elson spray for *N*-acetylhexosamines. $R_G$ values of methylated sugars refer to rates of movement relative to 2,3,4,6-tetra-*O*-methyl D-glucose in Solvent B. The butan-1-ol and pyridine used for preparative separations on Whatman No. 3MM sheets were purified by refluxing for 2 hours with KOH (1%, w/v) and distilling. Analytical reagent grades of other solvents were used without further purification.

Thin layer chromatography was carried out on plates coated with Silica Gel G or H as described previously (9). The plates were eluted by the ascending technique with the following solvent systems (v/v): (*a*) acetone-water-ammonia (250:3:1.5); (*b*) benzene-95% ethanol (20:5); (*c*) ethyl acetate-propan-1-ol-water (65:23:12). Sugars were detected with aniline-phthalate spray or by spraying with 25% $H_2SO_4$ and heating the plate for 30 min at 100°.

Electrophoresis was performed on both Whatman No. 1 and prewashed Whatman No. 3MM (for quantitative separation) paper with the following buffer systems: I, acetic acid-pyridine, pH 5.5; II, acetic acid-pyridine, pH 3.5; III, 0.05 N acetic acid-pyridine, pH 6.0. The same sprays as those described for paper chromatography were used for detection.

## EXPERIMENTS AND RESULTS

### *Methylation*

Nondialyzable keratosulfate (2.0 g) was initially acetylated as described for KS I (9). The dark brown liquid was poured with stirring into a large excess of ice-cold ethanol. The mixture was left in the refrigerator overnight, and the precipitate was separated by centrifugation. The precipitate was dissolved in water, dialyzed, and lyophilized, yielding 2.64 g (97%) of the acetylated product. This product was methylated twice with dimethyl sulfate and powdered NaOH in dimethylformamide (2.03 g; $OCH_3$, 16.4%) and once by Kuhn's procedure as described previously (9), and yielded 1.65 g (73.3%) of fully methylated KS II; $[\alpha]_D^{25}$ +5.9° (*c*, 1.73).

TABLE I

*Analysis of native and desulfated KS II used for methylation*

| Preparation | Galactose | Hexosamine | Sulfate | Fucose |
|---|---|---|---|---|
| | % | % | % | % |
| KS II | 31.4 | 28.6 | 18.7 | 2.5 |
| Desulfated KS II | 33.2 | 28.3 | 1.8 | <1.0 |

$C_{18}H_{31}O_{12}NSNa$

Calculated: $SO_4$ 16.6, $OCH_3$ 23.8
Found: $SO_4$ 17.6, $OCH_3$ 22.2

### *Hydrolysis of Methylated KS II*

The methylated product (1.50 g) was methanolyzed and then hydrolyzed with N $H_2SO_4$ for 7 hours. The hydrolysate after neutralization and concentration (9) yielded 1.64 g of methyl ethers.

### *Preparation of Desulfated Polymer*

Keratosulfate (200 mg) was desulfated as described under "Desulfation of Keratosulfates." The cloudy suspension was lyophilized without separation of the insoluble desulfated polysaccharide from the soluble fraction, to yield the desulfated product (103 mg; 68.7%) (Table I).

### *Methylation of Desulfated Polysaccharide*

The desulfated KS II was acetylated (125 mg) and methylated with dimethyl sulfate and powdered NaOH by the same procedure as that used for the native polysaccharide. The final product in this case was isolated by chloroform extraction, since the methylated, desulfated product was soluble in chloroform as opposed to the partial solubility of the methylated sulfated material. Yield, 52 mg; $OCH_3$, 26.4%. The partially methylated product (50 mg) was then methylated by Kuhn's procedure, extracted with chloroform, and precipitated by addition of light petroleum (b.p. 66–75°; yield, 41.3 mg).

$C_{19}H_{32}O_{10}N$

Calculated: $OCH_3$ 35.6
Found: $OCH_3$ 33.0

### *Hydrolysis of Methylated Desulfated KS II*

Trial hydrolysis was carried out with two 1-mg portions for optimal hydrolysis. The first portion was methanolyzed and then hydrolyzed with N $H_2SO_4$ for 6 hours at 100°. The second portion was added to 0.2 ml of ice-cold 72% $H_2SO_4$ and shaken for 1 hour at room temperature. The dark syrup was diluted with 1.6 ml of water to a final concentration of 3 N $H_2SO_4$ and heated at 100° until the reducing sugar value became constant (10, 11). The product was isolated after neutralization with

$BaCO_3$. Paper and thin layer chromatographic examination of the two products showed that the first procedure had caused some degradation. Hence the methylated, desulfated polysaccharide (39.5 mg) was hydrolyzed by the second procedure and yielded 36.3 mg of methyl ethers.

Paper chromatography in Solvents B and C and thin layer chromatography in Solvents *a* and *b* indicated that methyl ethers from methylated native KS II contained as major components 2,4,6-tri- and 2,4-di-*O*-methylgalactose and 3,6-di- and 3-*O*-methylglucosamine. The minor components were 2,3,4-tri-*O*-methylfucose and 2,3,4,6-tetra- and 2,3,4-tri-*O*-methylgalactose. In the case of the desulfated polymer, the methyl ethers were composed entirely of 2,4,6-tri- and 2,3,4,6-tetra-*O*-methylgalactose and 3,6-di-*O*-methylglucosamine.

### *Initial Separation*

The separation of neutral methylated sugars from methyl ethers of hexosamines was accomplished by passing an aqueous solution of the mixture of methyl ethers through a Dowex 50-$H^+$ column (30 × 2.5 cm). The eluate was recycled, and the column was washed with distilled water until the washings gave a negative anthrone test. The effluent was passed through an Amberlite IR-45 ($CO_3^=$) column, evaporated to a small volume, filtered, and dried. It yielded 654 mg of methylated neutral sugars. The Dowex 50 column was washed with an excess of 5% $NH_4OH$. The eluate was aerated to remove most of the ammonia, concentrated under reduced pressure to a small volume, filtered, and dried, to yield 542 mg of methylated hexosamine.

Similar fractionation of methyl ethers (32 mg) from the methylated, desulfated polysaccharide on a Dowex 50-$H^+$ column (20 × 1.5 cm) yielded methylated neutral sugars (11.5 mg) and methylated hexosamines (10.8 mg). The methylated neutral sugars from methylated native and methylated desulfated polysaccharides were converted to their methyl glycosides and examined by gas-liquid chromatography.[2] The results are summarized in Table II. The neutral sugar fractions (Fractions MN and MDN) and the methylated hexosamine fractions (Fractions MH and MDH) were examined by paper chromatography (Solvents A, B, and C) and thin layer chromatography (Solvents *a* and *b*). The results are given in Table III. The molar proportions of the sugars given for the native polysaccharide were approximated from the weights of the sugars isolated and, in the case of the desulfated polysaccharide, from paper, thin layer, and gas-liquid chromatography.

TABLE II

*Gas-liquid chromatographic examination of methyl glycosides of methylated neutral sugars from methylated native and desulfated KS II*

The results given here were obtained on a polyphenyl ether column at 185°. The samples were also analyzed on butanediol succinate ester at 190°; identical results were obtained.

| Sugar | Retention times | |
|---|---|---|
| | Native KS II | Desulfated KS II |
| | *min* | *min* |
| 2,3,4-Tri-*O*-methyl-L-fucose | 0.56 | |
| 2,3,4,6-Tetra-*O*-methyl-D-galactose | 1.17 | 1.15 |
| 2,4,6-Tri-*O*-methyl-D-galactose | 1.54; 1.70 | 1.55; 1.75 |
| 2,3,4-Tri-*O*-methyl-D-galactose | 2.29 (trace) | |
| 2,4-Di-*O*-methyl-D-galactose | 2.66; 3.20 | 3.20 (trace) |

TABLE III

*Approximate molar proportion of methyl ethers from KS II*

| Methyl ether | Desulfated polysaccharide | Native polysaccharide |
|---|---|---|
| 2,3,4,6-Tetra-*O*-methylgalactose | 0.5 | 1 |
| 2,4,6-Tri-*O*-methylgalactose | 10 | 8 |
| 2,3,4-Tri-*O*-methylgalactose | | 1 |
| 2,4-Di-*O*-methylgalactose | Trace | 10 |
| 2,3,4-Tri-*O*-methylfucose | | 1 |
| Unknown | | Present |
| 3,6-Di-*O*-methyl-*N*-methylglucosamine | 4 | 2 |
| 3,6-Di-*O*-methylglucosamine | 6 | 6 |
| 3-*O*-Methyl-*N*-methylglucosamine | | 4 |
| 3-*O*-Methylglucosamine | | 8 |

TABLE IV

*Fractionation of hydrolysate from methylated KS II*

| Fraction | Weight | $R_G$ | Probable identity of sugar |
|---|---|---|---|
| | *mg* | | |
| MN1 | 141.3 | 0.57 | 2,4-Dimethylgalactose[a] |
| MN2 | 18.6 | 0.77 | 2,3,4-Trimethylgalactose and unknown |
| MN3 | 132.6 | 0.80 | 2,4,6-Trimethylgalactose[a] and 2,3,4-trimethylfucose[a] |
| MN4 | 17.2 | 0.92 | 2,3,4,6-Tetramethylgalactose[a] |
| MH1 | 30.8 | 0.17 | 3-*O*-Methylglucosamine[a] |
| MH2 | 15.2 | 0.27 | 3-*O*-Methyl-*N*-methylglucosamine |
| MH3 | 2.8 | 0.27 + 0.31 | Mixture of MH2 and unknown |
| MH4 | 23.1 | 0.35 | 3,6-Di-*O*-methylglucosamine |
| MH5 | 6.9 | 0.52 | 3,6-Di-*O*-methyl-*N*-methylglucosamine |
| MH6 | 3.1 | 0.60 | Unknown |

[a] These compounds were characterized as crystalline derivatives.

### *Isolation of Methylated Monosaccharides*

The mixture (400 mg) of methylated neutral sugars from native KS II (Fraction MN) was applied to a charcoal-Celite column (30 × 2.5 cm), which was prepared as follows. Darco A-60 charcoal and Celite 535 were separately washed with concentrated HCl, followed by tap water, and finally with boiling distilled water. A water slurry of charcoal-Celite (1:1) was packed into a column which had a porous plate at the bottom and a pad of glass wool on the top. The methyl sugars were eluted with a gradient from 50% ethanol (750 ml) to water (750 ml). Fractions of about 10 ml were collected every hour. Aliquots of every third fraction, sometimes after concentration, were examined by paper chromatography. The appropriate tubes were combined to yield four fractions (MN1 to MN4) (Table IV).

*Fraction MN1*—The syrup, $[\alpha]_D$ +73.2° (*c*, 1.0), was indistinguishable from 2,4-di-*O*-methyl-D-galactose by paper and thin layer chromatography. Demethylation of a portion gave

[2] We are grateful to Dr. K. O. Lloyd of the Department of Microbiology, Columbia University College of Physicians and Surgeons, for carrying out these analyses.

galactose as the only monosaccharide. The syrup crystallized in the desiccator and was recrystallized from ethyl acetate; m.p. 96°. The aniline derivative was prepared by refluxing equimolar amounts of the sugar and redistilled aniline in absolute ethanol, with exclusion of light, for 30 min. The crystalline material after removal of the solvent was recrystallized from ethyl acetate; m.p. and mixed m.p. (with authentic 2,4-di-*O*-methyl-*N*-phenyl-D-galactosylamine) 213–214°.

*Fraction MN2*—Paper and thin layer chromatographic examination showed as the main component of this fraction 2,3,4-tri-*O*-methyl-D-galactose. In addition, small amounts of 2,4-di-*O*-methyl-D-galactose and an unknown ($R_G$ 0.79 in Solvent C) were present. Demethylation of a portion with boron tribromide followed by paper chromatography in Solvents G and I showed galactose as the main component. A small amount of a second component with the same mobility as that of mannose ($R_{Gal}$ 1.21 and 1.34 in the above two solvents) was present. It was difficult to decide whether this component was mannose or whether it was a monomethyl galactose from incomplete demethylation. A portion (1 mg) of the fraction was converted to the methyl glycosides and examined by gas-liquid chromatography. The main peaks corresponded to those of the glycosides of 2,3,4-tri-*O*-methyl galactose, in addition to a trace of an unknown peak.

*Fraction MN3*—The syrup (132.6 mg) was separated on Whatman No. 3MM sheets in Solvent C to give Fractions MN3a (109.4 mg) and MN3b (13.2 mg). MN3a, $[\alpha]_D$ +80.5° (*c*, 0.96), was indistinguishable from 2,4,6-tri-*O*-methyl-D-galactose by paper and thin layer chromatography. Demethylation with boron tribromide gave only galactose. The sugar (30 mg), converted to the aniline derivative and recrystallized as described for Fraction MN1, had a melting point of 197° and a mixed melting point (with authentic 2,4,6-tri-*O*-methyl-*N*-phenyl-D-galactosylamine) of 196–198°. MN3b, $[\alpha]_D$ −99.3° (*c*, 0.60), was identical with 2,3,4-tri-*O*-methyl-L-fucose on paper and thin layer chromatography. After conversion to its methyl glycoside, it gave a single peak identical with methyl 2,3,4-tri-*O*-methylfucoside on gas chromatographic analysis. Demethylation of a portion yielded fucose as the only monosaccharide. The sugar was finally converted into the aniline derivative; m.p. and mixed m.p. (with authentic 2,3,4-tri-*O*-methyl-*N*-phenyl-L-fucosylamine) 134–135°.

*Fraction MN4*—The main component of this fraction was 2,3,4,6-tetra-*O*-methyl D-galactose; traces of two other sugars were also present. The main sugar (14.6 mg), $[\alpha]_D$ +117.0° (*c*, 0.73), was obtained in pure form by fractionation on Whatman No. 3MM paper with Solvent C. After demethylation, galactose was the only monosaccharide detectable on paper chromatograms. Gas-liquid chromatography of its methyl glycosides on two different columns gave peaks identical with those of the methyl glycosides of authentic 2,3,4,6-tetra-*O*-methylgalactose. The syrup was converted to its aniline derivative and recrystallized from ether-petroleum ether; m.p. and mixed m.p. (with authentic 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine) 186–188°.

### *Fractionation of Methylated Hexosamines*

The mixture of methyl ethers of hexosamine (methylated hexosamines) (542 mg) from methylated native KS II was a dark syrup which streaked heavily on paper chromatograms. The syrup was dissolved in methanol and decolorized with active carbon to yield 402 mg of the purified sugars. A portion (300 mg) of this syrup was *N*-acetylated with acetic anhydride according to the method of Hirano, Hoffman, and Meyer (7), yielding 255 mg of product. Paper electrophoresis in System III revealed that *N*-acetylation was incomplete, since part of the sugar had the same mobility as that of glucosamine and the rest stayed at the origin as did *N*-acetylglucosamine. Hence the mixture was treated a second time with acetic anhydride. The resulting syrup was still incompletely *N*-acetylated and consisted of a variety of components (see "Discussion"). Hence the identification of the methylated hexosamines was carried out with the remaining one-quarter of the sample (102 mg). It was fractionated without *N*-acetylation on Whatman No. 3MM paper in Solvent B, and the six fractions (MH1 to MH6) were eluted from the paper with water and concentrated to syrups.

*Fraction MH1*—This fraction (30.8 mg; $R_G$ 0.17 in Solvent B) gave a single spot on paper chromatography in different solvent systems. Paper electrophoresis in Buffer III gave only one spot, with the mobility of glucosamine. The sugar reacted with aniline phthalate and ninhydrin spray but not with Morgan-Elson spray. Demethylation followed by ninhydrin gave only arabinose on paper chromatograms. A portion of the syrup was *N*-acetylated. The product had the same $R_F$ value as authentic 2-acetamido-2-deoxy-3-*O*-methyl-D-glucose. The *N*-acetylated product was crystallized from methanol and petroleum ether; m.p. and mixed m.p. (with crystalline 2-acetamido-2-deoxy-3-*O*-methyl-D-glucose obtained from KS I (9)) 156–158°.

*Fraction MH2*—This fraction (15.2 mg; $R_G$ 0.27 in Solvent B) also gave only one spot on paper chromatograms in Solvents B, D, and H. It reacted with aniline phthalate and alkaline silver nitrate and only faintly with ninhydrin spray. In contrast with Fraction MH1, it reacted slowly with the Morgan-Elson spray, giving a color characteristic of *N*-methylglucosamine (9). Ninhydrin treatment (12) of the fraction gave the same monomethyl arabinose as that obtained by similar tratment of Fraction MH1. On this basis, this fraction is 3-*O*-methyl-*N*-methylglucosamine.

*Fraction MH3*—This fraction (2.8 mg) gave two spots, with $R_G$ 0.27 and 0.31 in Solvent B. Thus it appeared to be a mixture of Fraction MH2 and an unknown. Demethylation of a portion followed by ninhydrin treatment gave arabinose, but no lyxose, as detected on paper chromatograms.

*Fraction MH4*—This fraction (23.1 mg; $R_G$ 0.35 in Solvent B) gave only one spot on paper chromatograms in Solvents B, D, and H. It reacted strongly with aniline-phthalate and ninhydrin, but not with Morgan-Elson spray, even after a long time. Demethylation with hydriodic acid followed by treatment with ninhydrin gave mainly arabinose, as seen by paper chromatography. Direct ninhydrin treatment of the fraction gave dimethyl arabinose (paper and thin layer chromatography), which was identical with that obtained by ninhydrin treatment of 3,6-dimethylglucosamine (obtained from methylated KS I). The product obtained by *N*-acetylation had the same mobility as authentic 2-acetamido-2-deoxy-3,6-di-*O*-methyl D-glucose.

*Fraction MH5*—This fraction (6.9 mg; $R_G$ 0.52 in Solvent B) was chromatographically and electrophoretically pure. It reacted with aniline-phthalate and alkaline silver nitrate, and faintly with ninhydrin spray; with Morgan-Elson spray it gave the characteristic reaction for an *N*-methylated hexosamine. Ninhydrin treatment of the fraction gave dimethyl arabinose, identical with that obtained from Fraction MH4. From these

reactions this fraction appears to be 3,6-di-*O*-methyl-*N*-methylglucosamine.

*Fraction MH6*—This fraction (3.1 mg; $R_G$ 0.60 in Solvent B) gave a single spot on paper chromatograms and paper electrophoresis. Its color reaction was similar to that of Fractions MH1 and MH4. Demethylation with hydriodic acid followed by ninhydrin gave mainly arabinose; no lyxose was detectable on paper chromatograms. Thus this is a methylated glucosamine and not a galactosamine derivative.

### *Examination of Methylated Sugars (Methylated, Desulfated Neutral Sugars and Hexosamines) from Desulfated KS II*

The methylated desulfated neutral sugars were examined by paper chromatography in Solvents B and C. In the former solvent, 2,4,6-tri-methyl galactose ($R_F$ 0.65) was the main sugar, with a smaller spot of 2,3,4,6-tetramethylgalactose ($R_F$ 0.77). In addition, traces of 2,4-dimethylgalactose and an unknown were present. In Solvent C, 2,4,6-trimethylgalactose ($R_F$ 0.20), 2,3,4,6-tetramethyl galactose ($R_F$ 0.60), and a trace of a spot with $R_F$ 0.05 were detected. Thin layer chromatography in Solvent *a* confirmed the presence of 2,3,4,6-tetramethylgalactose, 2,4,6-trimethylgalactose, traces of 2,4-dimethylgalactose, and an unknown with the same mobility as 2,3,4-trimethylgalactose. A sample of 2,4-dimethyl-3,6-anhydrogalactose was used on both paper and thin layer chromatograms as reference. In methylated, desulfated neutral sugars this sugar was absent.

Gas-liquid chromatography of the methylglycosides of methylated, desulfated neutral sugars was carried out with a butanediol succinate ester column at 190° and with a polyphenol ether column at 185° (13, 14). This analysis confirmed the presence of 2,4,6-trimethylgalactose (major), 2,3,4,6-tetramethylgalactose (minor), and 2,4-dimethylgalactose (trace). In addition, two very small peaks with $R_T$ 1.40 and 2.00 were detected in the first system. After demethylation of methylated desulfated neutral sugars (1 mg), galactose was the only free hexose detected on paper chromatograms run in Solvents G and I.

Paper chromatography of the methylated, desulfated hexos-, amines in Solvent B gave a strong spot of 3,6-dimethylglucosamine ($R_F$ 0.38), a smaller spot of 3,6-dimethyl-*N*-methylglucosamine ($R_F$ 0.46), and a trace of 3-*O*-methylglucosamine ($R_F$ 0.18). These spots gave characteristic color reactions with the aniline-phthalate, ninhydrin, and Morgan-Elson sprays. Paper chromatography in Solvent C gave only one very slow spot ($R_F$ 0.02). Similarly, on thin layer chromatography in Solvents *a* and *b*, this fraction was very slow and gave no useful information. The main product after ninhydrin oxidation was a dimethyl arabinose, paper chromatographically identical with that obtained by ninhydrin treatment of Fraction MH4. Arabinose was the only pentose detectable on paper after demethylation followed by ninhydrin oxidation.

### *Total Acid Hydrolysis of KS II*

Fraction MN2 from methylated native polysaccharide yielded, on demethylation, sugar with the chromatographic mobility of mannose, as stated earlier. Total acid hydrolysis of KS II followed by chromatography confirmed the presence of this sugar in the original preparation. Hence KS II (500 mg) was dissolved in 2 N $H_2SO_4$ (250 ml) and hydrolyzed by refluxing for 15 hours at 100°. The cooled solution was neutralized with a saturated solution of $Ba(OH)_2$, followed by addition of $BaCO_3$, and centrifuged. Concentration of the supernatant furnished a syrup (437 mg). A solution of this syrup was passed through successive columns of Dowex 50-$H^+$ and Amberlite IR-45 ($CO_3^=$). The columns were washed with water until the washings gave a negative test with anthrone. The effluent and water eluate were concentrated to a syrup, which was then fractionated into four fractions (A through D) on prewashed Whatman No. 3MM paper with Solvent A. The basic components were eluted from the Dowex column with 2 N $NH_4OH$. The eluate was aerated to remove ammonia and concentrated to a syrup. The two main components (Fractions E and F) were obtained in pure form by fractionation of this syrup on Whatman No. 3MM paper with Solvent G.

Fractions A and F were D-galactose and D-glucosamine, respectively, and were not investigated further. Fraction C (10.6 mg) was chromatographically (Solvents E, G, H, and I) and electrophoretically (borate buffer, pH 10.0) indistinguishable from fucose. The identification of L-fucose in KS II has been reported by Bray, Lieberman, and Meyer (15).

*Fraction B*—This fraction (7.7 mg; $[\alpha]_{final}$ +15.3° (*c*, 0.1)) was chromatographically identical with mannose in Solvents B, E, G, H, and I. Paper electrophoresis in borate buffer at pH 10.0 gave a single spot identical with mannose. The sugar (1.0 mg) was reduced with sodium borohydride, and the product was divided into two portions. One portion, examined paper chromatographically in Solvents G and I, showed spots identical with mannitol. The second portion of the reduced material was acetylated with acetic anhydride and pyridine, and the product was examined by gas-liquid chromatography on a column of ethylene glycol succinate combined with a cyanoethyl silicone column (16, 17) at 180° and 200°. The main peak ($R_{glucitol}$ 0.75) corresponded to that of mannitol hexaacetate. The sugar was finally converted to its phenylhydrazone (18); m.p. 193–195°; mixed m.p. (with an authentic sample of D-mannose phenylhydrazone) 196–198°.

*Fraction D*—This sugar (7.6 mg; $R_G$ 1.11, 1.62, and 1.24 in Solvents E, G, and I, respectively), on paper electrophoresis in Buffer I, stayed at the origin. It gave a brown spot, fluorescent in ultraviolet light, resembling the hexoses. The anthrone spray (19) specific for ketoses was positive. The identity of this fraction is unknown.

*Fraction E*—This fraction (18.2 mg; $[\alpha]_{D,final}$ +13.3° (*c*, 0.2); $R_{Gal}$ 0.35 and 0.45 in Solvents G and I, respectively) gave a positive reaction with ninhydrin and aniline-phthalate sprays. Hydrolysis of this sugar (10 mg) with 4 N HCl for 6 hours at 100°, followed by chromatography in Solvent I, showed galactose and glucosamine, while the major part of the fraction remained unhydrolyzed. Sodium borohydride reduction, followed by strong acid hydrolysis of the derived glycitol, gave no galactose but yielded glucosamine, galactitol, and some glucosaminitol. This sugar thus appeared to be a disaccharide of glucosamine and galactose, with the latter at the reducing end. A sample of the sugar was treated with a saturated solution of lime water for 27 hours at room temperature in an atmosphere of nitrogen. It was heated in a boiling water bath for 1 min. The cool solution was neutralized with phosphoric acid, calcium phosphate was removed by centrifugation, and the supernatant was concentrated to dryness. Paper chromatographic examination of the resulting syrup showed mainly glucosamine, with possibly a trace of mannosamine. Under the same conditions laminaribiose (G $1 \xrightarrow{\beta} 3$ G) gave glucose and a trace of mannose, and cellobiose

(G 1 $\xrightarrow{\beta}$ 4 G) gave only a little glucose and was mostly unchanged.

The sugar (2.0 mg) was converted to the methyl glycosides and oxidized with sodium periodate at room temperature for 6 hours. After addition of the calculated amount of ethylene glycol to destroy the excess periodate, it was hydrolyzed with N $H_2SO_4$ for 4 hours at 100°. The hydrolysate was neutralized with $BaCO_3$, concentrated, and examined by paper chromatography in Solvents G and I. Galactose was the only sugar detected on these chromatograms with silver nitrate and aniline-phthalate sprays.

### *Desulfation of Keratosulfates*

KS II (500 mg) was shaken with anhydrous methanol (50 ml) in a stoppered flask for 3 hours. Dry Dowex 50-X8 ($H^+$) (5 g) and dry NaCl (1 g) were added, and the heterogeneous mixture was shaken for 48 hours at room temperature. Distilled water (approximately 200 ml) was added to the flask, and the solution was stirred for 18 hours in the cold. The cloudy suspension was filtered through a pad of glass wool, and the resin remaining in the glass wool was washed twice with water, with stirring. The combined filtrates were dialyzed in the cold for 48 hours against stirred, distilled water. The contents of the bag were concentrated to a small volume and centrifuged. The precipitate was dispersed in distilled water and lyophilized to yield the water-insoluble Fraction DKS IIa (161 mg). The supernatant was lyophilized to yield the water-soluble Fraction DKS IIb (123 mg). The dialysate was concentrated to a syrup (Fraction DKS IIc).

Similarly, KS I (500 mg) was desulfated to yield the water-insoluble Fraction DKS Ia (212 mg) and the water-soluble Fraction DKS Ib (82 mg). The analyses of native and desulfated polysaccharides are given in Table V. Infrared spectra of the desulfated samples showed practically no sulfate peaks (1240 $cm^{-1}$, 850 $cm^{-1}$, and 820 $cm^{-1}$). Fractions DKS Ia and DKS IIa were insoluble also in dilute acid, alkali, borate buffer, 6 M urea, ethylene glycol, and 1-butanol. They swelled in formamide and dimethyl sulfoxide and dissolved on shaking. They were also soluble in strong acids (25% $H_2SO_4$, 4 N HCl). Fractions DKS Ia and DKS Ib did not cause any deepening in color of a dilute iodine solution. The amino acid compositions of KS I, DKS Ia, and DKS Ib were very similar. Digestion of Fraction DKS Ib with Pronase reduced the total amino acid content only slightly. About 44% of the remaining amino acid was aspartic acid, strongly supporting the proposed aspartyl linkage in KS I (6). Retreatment of a portion (50 mg) of the soluble Fraction DKS IIb with Dowex 50-$H^+$ and NaCl in methanol gave no additional insoluble Fraction DKS IIa. Gel filtration of DKS IIa and DKS IIb was carried out on a Sephadex LH-20[3] with 66% formamide as the eluent, in which both samples are soluble. Both samples were eluted in the void volume.

### *Examination of Dialyzable Fragments*

*Fraction DKS IIc*—This syrup was dissolved in water and desalted by passage through Dowex 50-$H^+$ and Amberlite IR-45 ($CO_3^=$) columns. The water eluate of the columns was concentrated to dryness, and the resulting syrup, on paper and thin layer chromatography, showed a number of spots reacting with silver nitrate, in addition to traces of free galactose and fucose. After total acid hydrolysis galactose, fucose, and glucosamine were detected in that order of intensity. Three fractions (DKS IIc1 through 3) were obtained by fractionation of 45 mg of DKS IIc on Whatman No. 3MM paper with Solvent B.

*Fraction DKS IIc1*—This fraction (32.5 mg), after initial examination on paper and thin layer chromatography, was further purified by thin layer chromatography on Silica Gel H plates with Solvent *c*. The sugar (9.2 mg) was identical with methyl fucoside on paper and thin layer chromatography. After hydrolysis with N $H_2SO_4$ for 3 hours, only fucose was detected on paper in Solvents D, E, F, and G.

*Fraction DKS IIc2*—Chromatography of 3.2 mg of Fraction DKS IIc2 in Solvent B with silver nitrate and aniline-phthalate sprays showed a spot with the mobility of fucose. However, after acid hydrolysis, fucose and some galactose were detected on paper. On paper chromatography in Solvent E, this fraction gave a double spot with mobilities corresponding to those of fucose and methyl galactoside. Thin layer chromatography in Solvent *c* showed two distinct spots with mobilities of fucose and methyl galactoside. Chromatography after sodium borohydride reduction and acid hydrolysis gave galactose and fucitol.

*Fraction DKS IIc3*—This fraction (2.5 mg) had the same mobility as *N*-acetyllactosamine on paper chromatograms and reacted with aniline-phthalate and silver nitrate sprays. On acid hydrolysis (2 N $H_2SO_4$, 7 hours, 100°), it gave only galactose and glucosamine. Sodium borohydride reduction followed by acid hydrolysis yielded galactose as the only reducing sugar.

TABLE V

*Desulfation of KS I and KS II*

Small amounts of mannose were detected in the total acid hydrolysates of both the water-soluble and water-insoluble fractions.

| Fraction | Yield | $[\alpha]_D$ in formamide | Sulfate | Galactose | Hexosamine | Fucose | Protein[a] |
|---|---|---|---|---|---|---|---|
| | % | | % | % | % | % | % |
| KS II | | −10.6 | 18.9 | 28.7 | 24.1 | 2.7 | N.D.[b] |
| DKS IIa | 42.9 | −11.1 | 1.0 | 34.5 | 29.4 | <1.0 | N.D. |
| DKS IIb | 32.6 | +9.6 | 1.5 | 30.4 | 27.4 | 1.1 | N.D. |
| KS I | | +1.12 | 17.7 | 35.3 | 26.2 | N.D. | 2.57 |
| DKS Ia | 56.4 | −6.6 | 0.6 | 45.0 | 42.4 | N.D. | 2.38 |
| DKS Ib | 21.8 | −5.9 | 1.5 | 35.9 | 27.7 | N.D. | 3.17 |

[a] By amino acid analysis.
[b] N.D., not determined.

## DISCUSSION

Old human rib cartilage keratosulfate used in the present studies was prepared by the usual procedure and was further purified by digestion with testicular hyaluronidase, followed by Pronase. The preparation had a low peptide content and was substantially free of chondroitin sulfate. It has been previously reported from this laboratory that KS II has a low molecular weight range and is partly dialyzable. The procedures used by us to isolate the methylated and desulfated polysaccharides included dialysis. The KS II preparation was dialyzed against stirred, distilled water for 48 hours in the cold room, and the non-dialyzable portion (approximately 90%) was used for the methylation and desulfation studies. KS II was acetylated and then methylated as described for KS I (9). The methylated KS II had a methoxyl content of 22.2%, which is 93% of the theoretical value expected on the basis of a monosulfated *N*-acetyllactos-

[3] Sephadex LH-20 was a gift from Pharmacia, Piscataway, New Jersey.

amine repeating unit. The yield of final product, 73%, was much better than the 42% yield obtained in the case of KS I. It is interesting that the methylated KS II had a positive optical rotation (+5.9°) as opposed to the starting material, which had a negative rotation (−7.2°). The desulfated KS II was methylated by the same method as for the native polysaccharide.

Preliminary paper and thin layer chromatography indicated that the hydrolysates from methylated native and methylated desulfated polysaccharides contained 3,6-dimethylglucosamine, 2,4,6-trimethylgalactose, and some 2,3,4,6-tetramethylgalactose. The hydrolysate from methylated native polysaccharide contained, in addition, 3-methylglucosamine, 2,4-dimethylgalactose, and some 2,3,4-trimethylgalactose. The methylated neutral sugars were separated from the methylated hexosamine by passing the mixture through a cation exchange agent. The methylated neutral sugars from both polymers were converted to their methyl glycosides and examined by gas-liquid chromatography in two different columns. The results were in full agreement with the paper and thin layer chromatography; *i.e.* the neutral sugar from methylated desulfated polysaccharide was mainly 2,4,6-tri-*O*-methyl-D-galactose, with some 2,3,4,6-tetra-*O*-methyl-D-galactose and only a trace of 2,4-di-*O*-methyl-D-galactose. The methylated native polysaccharide yielded 2,4,6-tri- and 2,4-di-*O*-methyl-D-galactose as the main components, in addition to smaller amounts of 2,3,6-tri-*O*-methyl-L-fucose and 2,3,4,6-tetra- and 2,3,4-tri-*O*-methyl-D-galactose. The mixture of methylated neutral sugars from the native polymer was fractionated on a carbon-Celite column. The following sugars were characterized by preparation of crystalline derivatives: 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl-D-galactose, and 2,3,4-tri-*O*-methyl-L-fucose. The composition of additional fractions is given in Table IV.

The major portion of the methylated hexosamines from the native polymer was *N*-acetylated by repeated treatment of the free bases with acetic anhydride. The product obtained appeared to be a complicated mixture, and fractionation proved difficult. The reason for this difficulty was probably the unsuspected presence of *N*-methylhexosamines in the mixture. As was shown earlier (9), *N*-methylhexosamines are not easily *N*-acetylated. This necessitated the repeated acetylations, which might even have caused some *O*-acetylation, thus giving rise to a wide variety of products. The methylated hexosamines were, however, identified after fractionation of the rest of the syrup on Whatman No. 3MM paper into six fractions.

One of the main components, 3-*O*-methyl-D-glucosamine, was characterized by its crystalline derivative. The others, 3-*O*-methyl-*N*-methyl-D-glucosamine, 3,6-di-*O*-methyl-D-glucosamine and 3,6-di-*O*-methyl-*N*-methyl-D-glucosamine, were identified by their chromatographic and electrophoretic behavior and by their color reactions. In addition, smaller amounts of two unidentified derivatives were also present. These components appeared to be glucosamine derivatives, rather than galactosamine, since both gave only arabinose after demethylation and ninhydrin treatment. The identities of the methylated sugar components from the desulfated polysaccharide are based on paper and thin layer chromatography, electrophoresis, and their reactions with spray reagents. The methylated neutral sugars were further identified by gas chromatography, and the methylated hexosamines by the products obtained on ninhydrin treatment. The results are summarized in Table III.

The desulfated polysaccharide appears to be a linear polymer of 3-linked galactose and 4-linked *N*-acetylglucosamine, probably in an alternating sequence. The nonreducing end groups are composed entirely of galactose residues, and the average chains appear to be made up of approximately 20 disaccharide units (Table III). In the case of the native polysaccharide, roughly 60% of the glucosamine derivatives are 3-methyl glucosamine and about 50% of the galactose derivatives are 2,4-dimethyl galactose. It thus appears that approximately half the galactose residues and about 60% of the glucosamine residues are substituted in position 6. The major portion of these substituents is sulfate. Since, during desulfation, fucose, sialic acid, galactose, and a minor quantity of glucosamine were lost due to methanolysis, it cannot be ruled out that the substituents at position 6 are carbohydrates. No methyl ether of fucose was found in the methylated, desulfated product. In the methylated native polysaccharide one fucose derivative, namely 2,3,4-trimethylfucose, was found. This indicates that all the fucose residues in KS II are present as terminal nonreducing groups. In spite of the fact that more galactose than glucosamine was lost during desulfation, 2,3,4,6-tetramethylgalactose is still present in the methylated, desulfated polysaccharide. This, together with the fact that no 3,4,6-trimethylglucosamine was detected either in the methylated native or methylated desulfated polysaccharide, indicates the presence of galactose branch points or consecutive galactose units in the main chain. The small amount of 2,3,4-trimethylgalactose in the methylated native polymer arises from terminal nonreducing galactose residues substituted at position 6. Again, the substituent could be sulfate or a sugar residue. Whichever is the case, the substituent must be acid-labile because it was removed during desulfation and no 2,3,4-trimethyl galactose was detected in the methylated, desulfated polysaccharide. The unknown fraction from methylated native polysaccharide gave, on demethylation, a sugar with the chromatographic mobility of mannose. However, the peaks on gas chromatography of the methyl glycoside of this fraction did not correspond to any of the known methylated mannose derivatives.

Complete characterization of methylated hexosamines was handicapped by the lack of reference sugars and also by the occurrence of partial *N*-methylation. However, the major components have been, at least tentatively, identified. About one third of the hexosamines was *N*-methylated (see Table III). *N*-Methylglucosamine can be determined quantitatively by analysis in the amino acid analyzer, where it appears between glucosamine and galactosamine under the conditions of hexosamine analysis at pH 5.28. The 6 N HCl hydrolysate of a fraction of methylated KS II showed almost 60% of the total hexosamine as *N*-methylglucosamine (uncorrected) by this method.

It is now well established (4, 6) that roughly about 10% of the hexosamine in KS II is *N*-acetylgalactosamine. Methylated galactosamine derivatives were not detected among the hydrolysis products of either the methylated native or the methylated desulfated polysaccharides. Bray, Lieberman, and Meyer (15) found that *N*-acetylgalactosamine in KS II is sensitive to alkali and that it is lost as the Kuhn chromogen, concomitant with the loss of serine and threonine, under very mild alkaline conditions. Hence it is very likely that the *N*-acetylgalactosamine was converted to the chromogen during the methylation (owing to the alkalinity) and destroyed on hydrolysis.

The isolation and identification of D-mannose from KS II was a surprise, since D-mannose has been considered typical of glycoproteins. Total hydrolysis of keratosulfates from different

sources, such as cornea, shark, nasal septum, and rib cartilage of Marfan's syndrome, also yielded mannose, as detected by paper chromatograms. Preparations purified by techniques such as free flow electrophoresis (20) still had mannose, thus eliminating the possibility of a contaminating glycoprotein in keratosulfate preparations, unless a contaminating glycoprotein was also sulfated. A KS II preparation reisolated after alkali treatment still contained mannose, which would exclude the possibility of its forming a glycosidic bond to the hydroxyamino acid. A disaccharide composed of galactose and glucosamine was also isolated from the total acid hydrolysate. This disaccharide was very resistant to acid hydrolysis and had galactose at the reducing end. Glucosamine was identified after alkaline elimination, suggesting a 1 → 3-linkage to galactose. This was further confirmed by the identification of only galactose after periodate oxidation and hydrolysis of the methyl glycoside of the disaccharide. The *N*-acetylated derivative of this disaccharide, with a sulfate group possibly in position 6 of the galactose, has been isolated by enzymic digestion of keratosulfate.[4] The isolation of these disaccharides and the isolation of sulfated *N*-acetyllactosamine (9) support the earlier propositions (7, 9) that D-galactose and D-glucosamine are linked in an alternating sequence in keratosulfate. The total hydrolysate also gave the expected galactose, glucosamine, and fucose. An unidentified trace component was present which had a faster chromatographic mobility in basic solvent as compared to acidic solvents and gave a positive reaction with a spray specific for ketose.

The procedures (21, 22) used for desulfation of sulfated polysaccharides such as chondroitin sulfate have been unsuitable for keratosulfate on account of extensive degradation and low yields. Hirano, Hoffman, and Meyer (7) modified these methods by using dry Dowex 50-$H^+$ and NaCl and succeeded in getting reasonable yields. However, when this experiment was repeated, it was found that part of the desulfated polysaccharide was insoluble in water.[5] The water-insoluble fraction was also insoluble in dilute acids, alkali, and a series of solvents listed in "Experimental Procedure." No significant difference in the carbohydrate or amino acid analysis was found that would explain the difference in the solubility of the two fractions. The slightly higher sulfate content (1.5%) of the water-soluble fraction probably does not explain its solubility, since further treatment of this fraction with the desulfating agent failed to produce the insoluble fraction. These fractions may differ in their molecular weights. The insolubility of one of the fractions prevented the conventional molecular weight determinations. On gel filtration on Sephadex LH-20, both fractions were excluded; however, Sephadex LH-20 has a very low exclusion limit.

In addition to the removal of sulfate ester groups, sialic acid, L-fucose, and, surprisingly, some galactose and *N*-acetyllactosamine were also cleaved by methanolysis during desulfation. Among the dialyzable fragments methyl fucoside, methyl galactoside, *N*-acetyllactosamine, and traces of galactose and fucose have been identified by chromatography. Sialic acid and L-fucose probably occupy terminal nonreducing positions in the carbohydrate chains, and the acid lability of such groups is not surprising. The preferential removal of galactose was surprising. Galactofuranose residues, which are acid-labile, could not be demonstrated in keratosulfate. The nature of the acid-labile galactosyl groups, probably at branch points, is at present unknown.

The main carbohydrate chains of both KS I and KS II are similar, namely, *N*-acetyllactosamine units polymerized through 1 → 3-linkages to galactose. In both cases the sulfate goups appear to be distributed in a random manner between positions 6 of D-galactose and *N*-acetyl-D-glucosamine, with the larger share of the sulfate groups on glucosamine. That branching occurs to a slightly greater extent in KS II is indicated by the presence of larger amounts of 2,3,4-trimethyl fucose and 2,3,4,6-tetramethyl and 2,3,4-trimethyl galactose in methylated KS II as compared to the traces of these components in methylated KS I (see "Discussion" in Reference 9). The actual size of the branches or the number of branches present is difficult to ascertain at this time. Our prediction is that sialic acid, fucose, and some galactose are present in the branches either individually or in small groups. Information along these lines is expected from the study of the oligosaccharides obtained by partial hydrolysis, acetolysis, and enzymatic hydrolysis of KS II.

[4] R. Lieberman and K. Meyer, unpublished results.

[5] Carragenan, a natural sulfated polygalactan, on desulfation remained entirely water-soluble.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 250, No. 7, Issue of April 10, pp. 2655-2659, 1975
*Printed in U.S.A.*

# Membrane-bound Neuraminidases of Rat Liver

## NEURAMINIDASE ACTIVITY IN GOLGI APPARATUS*

(Received for publication, August 14, 1974)

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### SUMMARY

**The bulk (60 to 65%) of the neuraminidase activity present in rat liver homogenates was found in the M + L (mitochondria plus lysosomes) fraction. The patterns of subcellular distribution were essentially identical whether disialogangliosides or neuramin-lactose (2→3′) were utilized as substrates.**

**A new neuraminidase, which hydrolyzes sialyl trisaccharides but which does not act upon glycoproteins and gangliosides, was detected in Golgi apparatus. Unlike the other particulate neuraminidases of rat liver, the Golgi enzyme is stimulated by prior incubation and by the addition of $Ca^{2+}$ or $Zn^{2+}$ at 1 mM concentration.**

**Although plasma membrane-rich fractions are often contaminated by Golgi membranes the marked differences in their enzymic properties allowed a clear distinction between the neuraminidases present in these two types of membranes.**

Rat liver contains soluble (2) and particle-bound neuraminidases (3). The soluble enzyme is a component of the cytosol (4), whereas the particulate enzyme was first found in lysosomes (3–7) and more recently in plasma membrane-rich fractions (8, 9). The observation that most of the particulate neuraminidase with specificity toward gangliosides appears to be associated with plasma membrane (8) whereas most of the neuraminidase activity measured with neuramin-lactose appears to be lysosomal (6) prompted distribution studies with both substrates. In addition, since plasma membrane preparations are often contaminated by Golgi membranes, neuraminidase activity was also investigated in highly purified Golgi preparations.

* This work was supported in part by United States Public Health Service Grant NS09176. A preliminary report has been published (1).

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### MATERIALS AND METHODS

Neuramin-lactose (2→3′) and neuramin-lactose sulfate (potassium salts) were prepared from rat mammary glands (10), while the 2→6′ isomer of neuramin-lactose was isolated from cow colostrum (11). Ovine submaxillary glycoprotein was kindly provided by the late Professor Alfred Gottschalk, Max Planck Institute for Virus Research, Tübingen, West Germany. Disialogangliosides were prepared from bovine brains as described by Winterbourn (12). The preparation utilized for this work contained 35.1% of *N*-acetylneuraminic acid (13); it had a molar ratio of *N*-acetylneuraminic acid to *N*-acetylgalactosamine (14) of 2.1 and 46% of its total sialic acid was released upon treatment with neuraminidase from *Clostridium perfringens*. Mixed gangliosides from bovine brain type II, 5′AMP, *N*-acetylneuraminic acid,[1] UDP-galactose (sodium salt), *N*-acetylglucosamine, glucose 6-phosphate, and neuraminidase type VI from *Clostridium perfringens* were products of Sigma. UDP-[$^{14}$C]galactose (274 mCi per mmol) was obtained from New England Nuclear. Fetuin from fetal calf serum and *p*-nitrophenyl thymidine 5′-phosphate were purchased from Calbiochem. All of the other chemicals utilized were of the highest purity commercially available.

Female Sprague-Dawley albino rats (220 to 260 g) fed *ad libitum* on a commercial pellet diet (Teklad Mills) were used for these studies.

The animals were stunned and killed by decapitation. The livers were quickly excised and chilled in ice-cold homogenizing solution; tissue homogenates were prepared and fractionated following published procedures as follows.

The method of Touster *et al.* (15) was adopted for the initial preparation of crude subcellular fractions and for the subsequent isolation of purified plasma membranes from the microsomal fraction.

Purified lysosomes were prepared by differential centrifugation as described by Ragab *et al.* (16).

For the isolation of Golgi apparatus, the method followed was essentially that of Leelavathi *et al.* (17). The Golgi fraction thus obtained was purified further by repeating the step of ultracentrifugation in discontinuous sucrose gradient. This procedure is similar to the one recently recommended by Dewald and Touster (18). The purified Golgi suspension was then diluted with distilled water to a sucrose concentration of 0.25 M and centrifuged for 30 min at 78,000 × *g*. The final Golgi pellet was gently suspended in glass-distilled water (1.8 mg of protein per ml) for neuraminidase assay. The Golgi marker enzyme, UDP-galactose: *N*-acetylglucosamine galactosyltransferase was assayed on a portion of the pellet suspended in a 0.5% (v/v) solution of Triton X-100.

The incubation mixture for the neuraminidase assay consisted of 135 μl of Golgi suspension, 15 μl of 1 M acetate buffer, pH 4.2, and 50 μl of substrate solution containing 200 nmol of bound sialic acid.

[1] The abbreviation used is: AcNeu, *N*-acetylneuraminic acid.

TABLE I

*Subcellular distribution of rat liver neuraminidase and of lysosomal and plasma membrane marker enzymes*

| Liver fractions[a] | Protein %[b] | 5′-Nucleotidase | | Phosphodiesterase I | | Acid phosphatase | | Neuraminidase[d] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | DSG | | NL | |
| | | RSA[c] | % | RSA | % | RSA | % | RSA | % | RSA | % |
| Nuclear | 20.6 | 0.91 | 18.7 | 1.02 | 21.1 | 0.66 | 13.6 | 0.83 | 17.2 | 1.04 | 21.4 |
| M + L | 28.0 | 0.35 | 9.7 | 0.64 | 17.8 | 1.86 | 52.1 | 2.11 | 59.2 | 2.36 | 66.1 |
| Microsomal | 21.6 | 2.99 | 64.5 | 2.58 | 55.7 | 0.77 | 16.6 | 0.77 | 16.6 | 0.42 | 9.1 |
| Soluble | 29.7 | 0.24 | 7.1 | 0.18 | 5.4 | 0.60 | 17.7 | 0.23 | 6.9 | 0.11 | 3.3 |
| | | | | | | | | 0.54[e] | 15.9 | 0.39[e] | 11.7 |

[a] Isolated by differential centrifugation (15).

[b] All percentages are based on total recovery. Actual over-all recoveries were within 10% of the values obtained with the unfractionated liver homogenate.

[c] RSA, relative specific activity: percentage of total activity divided by percentage of total protein of each fraction.

[d] Assayed using as substrate disialoganglioside (DSG) or neuramin-lactose 2 → 3′ (NL). Since all fractions contained 0.25 M sucrose, the incubation mixtures were fractionated by ion exchange chromatography prior to the determination of free *N*-acetylneuraminic acid.

[e] Values for the soluble neuraminidase assayed at pH 5.8.

The system was incubated for 2 hours at 37°. The reaction was stopped by the addition of 0.1 ml of periodate reagent (19) and the free sialic acid was assayed by a modification (20) of the thiobarbituric acid method of Warren (19). Alternatively, the enzyme reaction was stopped by quick freezing of the incubation mixture. After thawing, the samples were immediately subjected to ion exchange chromatography on columns (0.5 × 6 cm) of Bio-Rad AG 1-X2 (formate). The columns were first washed with distilled water and then eluted with 25 column volumes of 0.3 M formic acid. The eluates were lyophilized and free sialic acid was then assayed in the residue dissolved in distilled water. Enzyme and substrate controls were incubated concurrently and their values subtracted from the experimental values. Neuraminidase assays in other particulate fractions and in the soluble fraction were done by the same procedure but at their pH optima, 4.4 and 5.8, respectively (4).

All other enzyme assays were done by published methods as follows. UDP-galactose:*N*-acetylglucosamine galactosyltransferase was assayed by the methods of Fleischer and Fleischer (21) and of Morre *et al.* (22); 5′-nucleotidase and phosphodiesterase I were determined as described by Touster *et al.* (15); acid phosphatase was measured according to Berthet and de Duve (23); α-D-mannosidase was assayed at pH 5.5 by the method of Dewald and Touster (18) and glucose-6-phosphatase was determined according to de Duve *et al.* (24).

Protein was determined according to Lowry *et al.* (25) using crystalline bovine serum albumin as the standard.

For ultrastructural studies, the pelleted Golgi fractions were fixed in 2% glutaraldehyde and postfixed with osmium tetroxide. After washing with buffered normal formalin, the material was dehydrated in graded ethanol and embedded in Araldite 6005. Sections of these pellets were examined in a Hitachi HU-11B electron microscope.

## RESULTS

The enzyme content of liver subcellular fractions obtained by differential centrifugation (15) shows that the bulk of the neuraminidase activity and the highest relative specific activity was recovered in the combined mitochondria plus lysosomes (M + L) fraction (Table I) whether disialogangliosides or neuramin-lactose (2→3′) were used as the substrate. The distribution of neuraminidase resembles that of the lysosomal marker enzyme, acid phosphatase, while the plasma membrane marker enzymes, 5′-nucleotidase and phosphodiesterase I, appeared concentrated in the crude nuclear and microsomal fractions as expected. The microsomal pellet was fractionated by discontinuous sucrose-gradient centrifugation (15) and the same four enzymes were assayed in the isolated subfractions $P_2$, $P_3$, and $P_4$. In general agreement with a previous report (8), all of the enzymes exhibited their highest relative specific activity in the plasma membrane-rich fraction ($P_2$), which collects at the 0.25 M-34% sucrose interface. This fraction was the only one that consistently gave measurable levels of neuraminidase activity towards both neuramin-lactose and disialogangliosides (Table II). Release of sialic acid from endogenous substrates (8) was also demonstrated; the product of this hydrolysis was characterized as *N*-acetylneuraminic acid by paper chromatography (26).

TABLE II

*Neuraminidase activity in rat liver plasma membrane*

Fraction $P_2$ isolated from the microsomal pellet (15) and suspended in distilled water.

| Substrate | AcNeu released[a] |
|---|---|
| | *nmol/hr/mg protein* |
| Neuramin-lactose (2→3′) | 6.70 |
| Disialogangliosides | 3.42 |
| Endogenous substrates[b] | 3.02 |

[a] All incubation mixtures were fractionated by ion exchange chromatography.

[b] Membrane fraction incubated at 37° for 90 min in 0.1 M acetate buffer, pH 4.4, and centrifuged for 30 min at 105,000 × *g*. Supernate used to measure sialic acid released from endogenous substrates.

Since it was recently reported that plasma membranes prepared by this method are contaminated by variable amounts of Golgi membranes (18), plasma membranes ($P_2$) and Golgi-rich fractions were prepared and assayed for neuraminidase and several marker enzymes. The results obtained for the marker enzymes (Table III) are in close agreement with those reported in the literature (18).

The specific activities of neuraminidase and of the Golgi marker enzymes α-mannosidase and galactosyltransferase are considerably higher (315%, 337%, and 378%, respectively) in Golgi than in plasma membrane. Conversely, the specific activities of the plasma membrane markers (5′-nucleotidase and phosphodiesterase I), of the lysosomal marker (acid phosphatase) and of the microsomal marker (glucose 6-phosphatase) in

TABLE III

*Comparison of levels of various enzymes in preparations of plasma membrane and Golgi apparatus from rat liver*

| Enzyme | Plasma membrane[a] | Golgi apparatus[b] |
|---|---|---|
| | *Enzyme units[c] per mg protein* | |
| 5′-Nucleotidase | 1.67 | 0.26 |
| Phosphodiesterase I | 1.03 | 0.24 |
| Galactosyltransferase[d] | 2.47 | 9.34 |
| α-Mannosidase | 6.21 | 20.94 |
| Neuraminidase[e] | 4.43 | 13.97 |
| Acid phosphatase | 22.34 | 10.63 |
| Glucose 6-phosphatase | 0.15 | 0.07 |

[a] Prepared according to Touster *et al.* (15); yield 1.48 mg of protein per g of liver.

[b] Prepared according to Dewald and Touster (18); yield 0.62 mg of protein per g of liver.

[c] For 5′-nucleotidase, phosphodiesterase I, and glucose-6-phosphatase, 1 enzyme unit corresponds to the activity hydrolyzing 1 μmol of substrate per min; for galactosyltransferase, α-mannosidase, and acid phosphatase units are expressed in nanomoles per min and for neuraminidase in nanomoles per hour.

[d] Determined by the method of Fleischer and Fleischer (21).

[e] Determined with neuramin-lactose (2→3′) as the substrate.

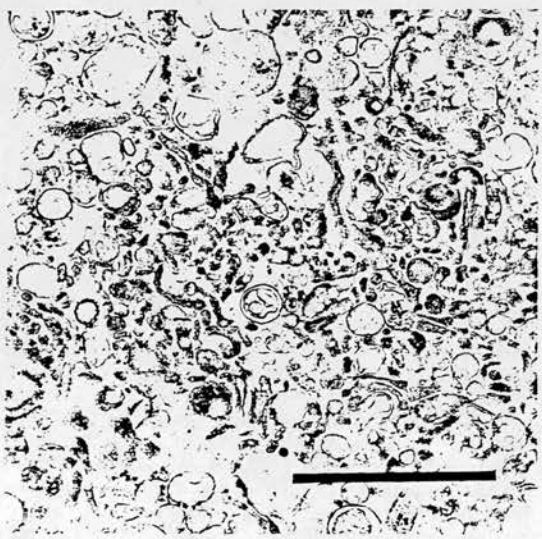

FIG. 1. Electron micrograph of a rat liver Golgi fraction. The bar represents 1 micrometer.

Golgi are less than 50% of the values found in plasma membrane. These results provide strong support for the presence of neuraminidase activity as an integral part of the Golgi membranes.

Further characterization of the Golgi neuraminidase was conducted on preparations obtained as described under "Materials and Methods." The high purity of these preparations was ascertained by the high specific activity of the galactosyltransferase (22) (248 to 343 nmol per hour per mg of protein) and by occasional examination by electron microscopy (Fig. 1). The specific activity for Golgi neuraminidase usually ranged between

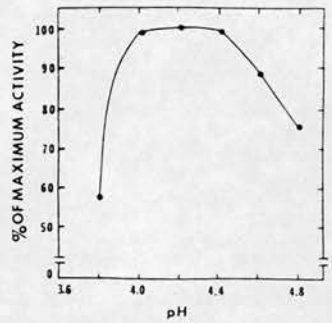


FIG. 2. pH *versus* activity curve of rat liver Golgi neuraminidase. The enzyme activity was measured with neuramin-lactose (2→3′) as the substrate in the presence of sodium acetate-acetic acid buffers (75 mM final concentration).

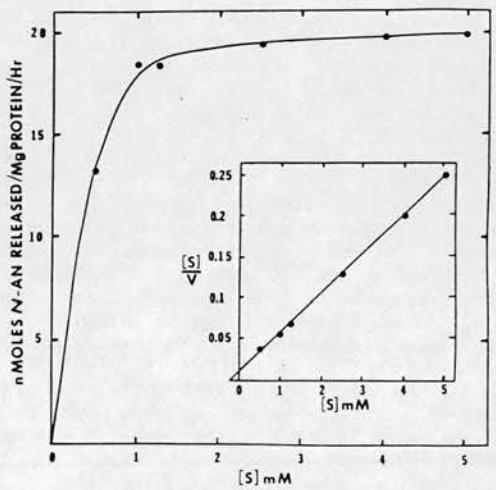


FIG. 3. Effect of the concentration of neuramin-lactose (2→3′) on the rate of hydrolysis. *N*-AN, *N*-acetylneuraminic acid.

10 and 25 nmol per hour per mg protein, although values as high as 150 have been observed.

The pH value *versus* activity curve of Golgi neuraminidase, studied with acetate buffer, displayed a broad peak between pH 4.0 and 4.4 with a maximum at about pH 4.2 (Fig. 2). The rate of hydrolysis of neuramin-lactose (2→3′) was linear for up to 2 hours of incubation at 37°. The reaction velocity was also linear with increasing enzyme concentrations between 0.06 and 0.55 mg of protein. The effect of substrate concentration (neuramin-lactose, 2→3′) on the rate of hydrolysis is shown in Fig. 3; the $K_m$ calculated from the plot $[S]/v$ *versus* $[S]$ (27) gave a value of $2.17 \times 10^{-4}$ M. Stimulation of the enzymic activity was noticed upon preincubation at 37° in acetate buffer, pH 4.2, for 1 hour. The magnitude of the stimulation varied (between 50 and 80%) from preparation to preparation. Preincubation of lysosomes isolated by differential centrifugation (16) or of Triton WR-1339-filled lysosomes (4) under similar conditions resulted in a 30 to 50% loss of neuraminidase activity.

The effect of several divalent cations (as their chlorides) on the enzyme activity was also studied. Addition of $Ca^{2+}$ or $Zn^{2+}$

TABLE IV

*Hydrolysis of various substrates by rat liver Golgi neuraminidase*

| Substrate | AcNeu released |
|---|---|
| | *nmol/hr/mg protein* |
| Neuramin-lactose (2→3′) | 11.00 |
| Neuramin-lactose (2→6′) | 6.36 |
| Neuramin-lactose sulfate | 34.23 |
| Fetuin | 0 |
| Ovine submaxillary glycoprotein | 0 |
| Disialogangliosides | 0[a] |

[a] Differential spectrum (complete enzyme system recorded against enzyme blank) of the chromogens obtained by direct thiobarbituric acid assay of the incubation mixtures showed an interfering peak with $\lambda_{max}$ at 535 nm. Fractionation of the incubation mixtures by ion exchange chromatography prior to the thiobarbituric acid assay (see "Materials and Methods") revealed absence of free AcNeu, indicating lack of hydrolysis of disialogangliosides.

at 1 mM concentration resulted in about 40% activation, while $Hg^{2+}$ ions at the same concentration caused 40 to 50% inhibition of the enzyme. $Cu^{2+}$ at 1 mM concentration had no effect on the enzyme activity.

Higher concentrations of chlorides of mono and divalent cations ($Na^+$, $K^+$, $Li^+$, and $Ca^{2+}$) were inhibitory to the enzyme. At 200 meq per liter, only 40 to 50% of the initial activity was detected.

The rate of hydrolysis of different substrates by Golgi neuraminidase was also studied. Neuramin-lactose sulfate was hydrolyzed much faster than neuramin-lactose (2→3′) which in turn was a much better substrate than the 2→6′ isomer of neuramin-lactose. Golgi preparations showed no neuraminidase activity towards macromolecular substrates such as fetuin, ovine submaxillary glycoprotein, and gangliosides under the experimental conditions described (Table IV).

A point still under study, but which nevertheless should be mentioned at this time, is that Golgi preparations obtained from the livers of female rats, in general, appear to have higher neuraminidase activity than those obtained from male rats.

## DISCUSSION

There are marked discrepancies between the results reported from different laboratories regarding the relative distribution of particulate neuraminidase in rat liver subcellular fractions. Using neuramin-lactose as the substrate, Horvat and Touster (6) observed that the lysosomal fraction was the richest (47%) while only 32% of the total activity was recovered in the microsomal fraction. On the other hand, Schengrund *et al.* (8), using ganglioside as the substrate, found 54% of the total neuraminidase activity in the microsomal fraction and only 26% in the combined mitochondrial-lysosomal pellet. In our studies on the intracellular distribution of neuraminidase, the major portion of the particulate neuraminidase activity was found in the M + L fraction, regardless whether neuramin-lactose or disialogangliosides were used as substrates (Table I). The reasons for these differences are not readily apparent and they are probably due to the combined effect of differences in the *modus operandi* and in the composition of the substrates. Commercial neuramin-lactose is usually isolated from cow colostrum and it contains a mixture of the 2→3′ and 2→6′ isomers (11), whereas the neuramin-lactose utilized for our studies is pure 2→3′ isolated from rat mammary glands (10). Differences in the rates of hydrolysis obtained with these two isomers are quite significant (Table IV) and variations in the relative proportions of these two components in different specimens might possibly account for the fact that, while some authors consider neuramin-lactose to be a relatively poor substrate (8), other investigators find it satisfactory (9), and, we found it more sensitive (Table I) than gangliosides for all of the enzyme preparations assayed in these studies. The possible effect of variations in the relative proportions of the individual gangliosides present in commercial mixed gangliosides and in disialogangliosides is another factor that needs to be evaluated. A technical point that, unless adhered to, can invalidate the results is that the assay of the sialic acid liberated by plasma membrane preparations can be conducted only after fractionation of the incubation mixture by ion exchange chromatography because spectroscopic analysis of the chromogens obtained by direct thiobarbituric acid assay on the incubation mixtures gave a differential spectrum (*i.e.* spectrum of the complete enzyme system recorded against the enzyme blank) with $\lambda_{max}$ at 535 nm instead of the typical peak at 549 nm (19).

In spite of the differences in enzyme distribution described above, our findings (Table II) are consistent with previous reports (8, 9) concerning the presence of neuraminidase activity toward endogenous and exogenous substrates in plasma membrane-rich fractions.

The fact that the neuraminidase activity found in plasma membranes is not due to lysosomal contamination has been clearly substantiated (9). On the other hand, since plasma membrane fractions prepared by the method of Touster *et al.* (15) are contaminated with Golgi membranes (18), the Golgi neuraminidase described in this paper may contribute, in some measure, to the neuraminidase activity assayed with neuramin-lactose in plasma membrane preparations.

The three membrane-bound neuraminidases of rat liver (lysosomal, plasma membrane, and Golgi) differ markedly in some of their properties, suggesting that they are probably three different enzymic entities. As indicated above (Table IV), the Golgi neuraminidase does not hydrolyze gangliosides which in turn are good substrates for lysosomal (4–6) and plasma membrane (8–9) neuraminidases. While preincubation of Golgi preparations causes 50 to 80% stimulation of the activity towards neuramin-lactose, no such stimulation was observed with plasma membranes (9) and a marked inhibition was observed with tritosomes (4) and with lysosomal preparations obtained by differential centrifugation (16). Using neuramin-lactose as the substrate, the $K_m$ for the Golgi neuraminidase was $2.17 \times 10^{-4}$ M, whereas the values reported for the lysosomal and plasma membrane neuraminidases (9) were $1.27 \times 10^{-3}$ M and $6.86 \times 10^{-4}$ M, respectively. Marked differences in the behavior of these three enzyme preparations were also observed in the presence of 1 mM concentration of several divalent cations. While $Ca^{2+}$, $Zn^{2+}$, and $Hg^{2+}$ had little or no effect on the lysosomal enzyme (4), $Ca^{2+}$ and $Zn^{2+}$ stimulated the Golgi enzyme by 40% while $Hg^{2+}$ caused 40 to 50% inhibition. $Cu^{2+}$, on the other hand, caused more than 90% inhibition of the plasma membrane neuraminidase (9) but had no effect on either the lysosomal (4) or the Golgi enzymes.

In addition to neuraminidase, two other glycohydrolases ($\alpha$-mannosidase and *N*-acetyl-$\beta$-glucosaminidase) have been detected (18) in Golgi apparatus. The presence of these degradative enzymes in an organelle which appears to be the major site for glycosyltransferases involved in biosynthetic processes is not to be regarded as an incongruity. Actually, these findings support

current views concerning the participation of the Golgi apparatus in the biogenesis of lysosomes. Since the Golgi neuraminidase, unlike its lysosomal counterpart, does not attack macromolecular substrates, this form of the hydrolase is compatible with the presence of newly synthesized sialoglycoconjugates within the same organelle. The other two glycohydrolases found in Golgi, *i.e.* α-mannosidase and *N*-acetyl-β-glucosaminidase, have been assayed using low molecular weight synthetic substrates, and hence it would be of considerable interest to establish whether they are active or inactive toward glycoproteins.

In the case of neuraminidase and α-mannosidase, marked differences have been observed between the enzymic properties of the respective Golgi and lysosomal enzymes; no data is yet available for *N*-acetyl-β-glucosaminidase. These differences in properties and specificity may be caused by intrinsic structural changes or by changes in the microenvironment of these enzymes as the endomembrane differentiation from Golgi vesicles to lysosomes shifts these glycohydrolases from a biosynthetic subcellular compartment to one committed exclusively to a degradative function or both. Studies with highly purified hydrolases from Golgi apparatus and from lysosomes should help elucidate possible structural and functional interrelationships between these homologous enzymes.

*Acknowledgments*—The authors wish to express their appreciation to Anna Henley and to Timothy Poole for their technical assistance. The ultrastructural studies were performed through the courtesy of Dr. Robert E. Nordquist.

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ANALYTICAL BIOCHEMISTRY 69, 385–394 (1975)

# Neuraminidase Assay Utilizing Sialyl-Oligosaccharide Substrates with Tritium-Labeled Aglycone

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Received March 21, 1975; accepted July 7, 1975

A new method for the radioisotopic assay of neuraminidase activity has been developed. The substrate utilized, α-D-*N*-acetylneuraminosyl-(2 → 3′)-lactit[$^3$H]ol, was prepared by reduction of α-D-*N*-acetylneuraminosyl-(2 → 3′)-lactose with tritiated borohydride and purified by ion-exchange chromatography. After incubation with neuraminidase, the reaction mixtures were applied to small columns of AG 1-X2 (formate) in order to remove free sialic acid and unhydrolyzed substrate. The lactit[$^3$H]ol released by neuraminidase action was then recovered by washing the columns with distilled water and quantitated by utilizing a liquid scintillation spectrometer. Studies with bacterial, avian, and mammalian neuraminidases are described.

Neuraminidases are widely distributed (1) in microorganisms (viruses, mycoplasmae, and bacteria) as well as in animal tissues (amphibian, avian, and mammalian). The enzyme activity is usually assayed by measuring the sialic acid released from natural substrates (oligosaccharides, gangliosides, and glycoproteins). The thiobarbituric acid[2] methods (2,3), widely used for the estimation of free sialic acids, often do not give reliable results with crude enzyme preparations. Substances such as deoxyribose (2), sucrose (from the homogenizing solutions) (4), lipid peroxides, 3-deoxyaldulosonic acid (5), and unsaturated uronic acids (6) are some of the compounds known to interfere with this assay. In addition, and for reasons not quite understood, several compounds containing bound sialic acids have been reported to give a positive TBA test (7–9). In some cases, suitable corrections can be calculated from dichromatic readings (2) but in many instances, isolation of the free sialic acid must be conducted prior to the TBA assay (4).

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[2] Abbreviations used: TBA, thiobarbituric acid; NL(2 → 3′), α-L-*N*-acetylneuraminosyl-(2 → 3′)-lactose; NL(2 → 6′), α-D-*N*-acetylneuraminosyl-(2 → 6′)-lactose; NL[$^3$H]ol, α-D-*N*-acetylneuraminosyl-(2 → 3′)-lactit[$^3$H]ol.

The new method described in this communication circumvents these problems by utilizing as the substrate a sialyl-oligosaccharide with a neutral radioactively labeled aglycone. Free sialic acid and unhydrolyzed substrate are removed from the reaction mixture with an anion-exchange resin and the aglycone released by the enzyme action is then measured by scintillation spectrometry.

## MATERIALS AND METHODS

Neuramin-lactose was isolated from skimmed bovine colostrum by ion-exchange chromatography, essentially as described by Schneir and Rafelson (10). It should be pointed out, however, that this method gave only a partial separation of NL(2 → 3′) from NL(2 → 6′). In order to complete the isolation of pure NL(2 → 3′) utilized for these studies, the intermediate fractions, containing a mixture of the two isomers, were further fractionated by preparative paper chromatography. This separation was performed on prewashed Whatman No. 3MM paper by descending irrigation with ethyl acetate:pyridine:acetic acid:water (5:5:1:3, by volume). $^3$H-labeled sodium borohydride and Aquasol were purchased from New England Nuclear. Neuraminidase (type VI) from *Clostridium perfringens* and crystalline synthetic *N*-acetylneuraminic acid (type IV) were products of Sigma. All other chemicals were obtained commercially and they were the highest purity available.

Total sialic acid was determined by the periodate–resorcinol method (11). Free sialic acid was measured by the TBA assay of Warren (2) as modified by Yeh *et al.* (12). Protein was determined by the method of Lowry *et al.* (13) with crystalline bovine serum albumin as the standard. Lactose and lactitol were assayed by the anthrone reaction (14).

Radioactivity was measured in a Nuclear-Chicago Mark I liquid scintillation system using a mixture of 1 ml of aqueous sample and 12 ml of Aquasol. The efficiency was about 17%.

## RESULTS

### *Preparation of Tritium-Labeled Neuramin-Lactitol*

A solution of $NaB[^3H]_4$ (231 μmoles, total activity 7.29 mCi) in 2 ml of 0.01 N NaOH was added to 67 μmoles of NL(2 → 3′)(potassium salt) and the reaction was allowed to proceed at room temperature in a stoppered test tube with occasional shaking. At the end of 2.5 hr, $NaBH_4$ (20 mg) in 0.5 ml of 0.01 N NaOH was added and the reaction was allowed to proceed for an additional 4 hr. The reaction was then stopped by decomposing the excess borohydride by dropwise addition of acetone (1 ml). The solution was applied to a Bio-Rad AG 50-X2 ($H^+$) column (0.9 × 7 cm) and eluted with 60 ml of distilled water. This operation was done in a cold room (4°C) since earlier experience in-

TABLE 1
ANALYSIS OF α-D-*N*-ACETYLNEURAMINOSYL-(2 → 3′)-LACTIT[$^3$H]OL

| Component | Percentage of dry weight | | Method |
|---|---|---|---|
| | Calculated[a] | Found | |
| Total *N*-acetylneuraminic acid | 43.0 | 37.7 | Periodate–resorcinol (11) |
| Free *N*-acetylneuraminic acid | 0.0 | 2.8 | Thiobarbituric acid (2) |
| Lactit[$^3$H]ol | 51.2 | 45.8 | Anthrone[b] (14) |
| Potassium | 5.8 | n.d.[c] | — |

[a] Calculated from molecular weight of 675.
[b] Lactitol was used as the standard.
[c] n.d., Not determined.

dicated that some hydrolysis of neuramin-lactitol occurred when it was exposed to this cation-exchange resin at room temperature. The effluent and washings from the column were evaporated to dryness under reduced pressure at temperatures under 30°C. Borate was eliminated as volatile methyl borate by subjecting the residue to repeated evaporation (about five times) with methanol under reduced pressure. In order to remove the "acid-stable" radioactive impurities (15) the final residue was dissolved in 2 ml of distilled water and applied to a Bio-Rad AG 1-X2 (formate) column (1.3 × 23 cm) at 4°C. The column was washed with an excess (300 ml) of distilled water and then eluted with 0.01 M pyridinium formate (16); the eluate was collected in 20-ml fractions. The fractions corresponding to a sharp peak of material reacting positively with the resorcinol reagent (11) and showing a high tritium count were pooled and lyophilized. The syrupy residue was then dissolved in 1 ml of water and passed through a column (0.6 × 7 cm) of Bio-Rad AG 50-X2 ($H^+$). The effluent and washings were neutralized with 0.05 M KOH and lyophilized to yield 45.3 mg of the potassium salt of NL[$^3$H]ol with a specific radioactivity of 0.73 μCi/μmole. The chemical analysis (Table 1) indicated a recovery of 59 μmoles of product with a purity of 90% and small amounts of TBA-reacting material. A single spot ($R_{N\text{-acetyl neuraminic acid}}$ 0.59; $R_{NL(2\to3')}$ 0.89; $R_{lactitol}$ 0.50) was observed on paper chromatograms developed for 40 hr with the solvent system described under methods. The spot could be visualized with periodate–benzidine (17) or with alkaline silver nitrate (18) but, as expected, it failed to react with the aniline–oxalate (19) spray reagent for reducing sugars. When a 4-μmole sample was subjected to mild acid hydrolysis (0.1 N $H_2SO_4$, 80°C, 1 hr) and the hydrolysate was neutralized with $BaCO_3$, centrifuged, and passed successively through coupled columns (0.9 × 7 cm) of AG 50-X2 ($H^+$) and AG 1-X2 (formate), the anthrone-

positive material and the tritium counts were quantitatively recovered in the water eluate. Elution of the AG 1-X2 (formate) column with 0.3 *N* formic acid yielded the free sialic acid and less than 1% of the initial radioactivity.

The potassium salt of NL[$^3$H]ol appears to be as stable as its parent compound NL(2 → 3′), since essentially no decomposition was observed after storage for 11 months at −20°C.

*Neuraminidase Studies*

The incubation system utilized for the neuraminidase assay (12) consisted of 135 μl of enzyme preparation, 15 μl of 1 M sodium acetate–acetic acid buffer (pH value indicated in each experiment) and 200 nmoles (ca. $3.2 \times 10^5$ dpm) of tritium-labeled neuramin-lactitol dissolved in 50 μl of distilled water. The mixtures were incubated at 37°C for various lengths of time (from 30 to 180 min). Control tubes containing heat-inactivated enzyme, substrate, and buffer were incubated concurrently. At the end of the incubation, the reaction mixtures were quantitatively transferred to columns (0.6 × 7 cm) of AG 1-X2 (formate). After washing the column with an excess (20 ml) of distilled water, the combined eluate was lyophilized and the residue was dissolved in 2 ml of water. An aliquot, usually 1 ml, was mixed with Aquasol for radioactivity measurements. One unit of neuraminidase activity is defined as the amount of enzyme that hydrolyzes 1 nmole of substrate per hour under the conditions of the assay. Specific activities are expressed as units of neuraminidase activity per milligram of protein.

For initial enzymic studies, a dilute (500 μg/ml) aqueous solution of *Clostridium perfringens* neuraminidase was utilized and the incubations were conducted for 1 hr at pH 5.0. The radioactive lactit[$^3$H]ol was isolated from the incubation mixture by the anion-exchange column described above, by paper chromatography, and by paper electrophoresis (0.1 M ammonium acetate–acetic acid buffer, pH 4.65, 375 V for 4 hr, on Schleicher and Schuell paper No. 598). In the last two experiments, the lactit[$^3$H]ol position was detected by spraying strips containing duplicate samples run concurrently, and the paper areas containing the lactit[$^3$H]ol spot were cut out, placed in vials with Aquasol and counted. The enzyme activity was also estimated by the assay of the sialic acid released by direct TBA assay of the incubation mixture or by assay of the sialic acid eluted with 0.3 N formic acid from the same columns utilized for the isolation of radioactive lactitol. As shown in Table 2, the results obtained agreed closely among themselves and with the results obtained by the usual procedure, i.e., direct TBA assay using NL(2 → 3′) as the substrate.

TABLE 2

ASSAY OF BACTERIAL NEURAMINIDASE[a] UTILIZING AS SUBSTRATES α-D-*N*-ACETYLNEURAMINOSYL(2→3′)LACTOSE AND α-D-*N*-ACETYLNEURAMINOSYL(2→3′)LACTIT[$^3$H]OL

| | *N*-Acetylneuraminic acid (nmoles/hr/mg protein) | | Lactit[$^3$H]ol (nmoles/hr/mg protein) | | |
|---|---|---|---|---|---|
| | | | Isolated from the incubation mixture by | | |
| Substrate | Direct TBA Assay | TBA after isolation by ion-exchange chromatography | Ion-exchange chromatography | Paper chromatography | Paper electrophoresis |
| NL[$^3$H]ol[b] | 13,152 | 11,560 | 13,840 | n.d.[d] | n.d. |
| NL[$^3$H]ol[b] | 12,584 | 11,032 | 13,464 | 11,680 | 11,232 |
| NL(2→3′)[c] | 13,204 | n.d. | — | — | — |

[a] Neuraminidase from *Clostridium perfringens* (Sigma, type IV).
[b] NL[$^3$H]ol, α-D-*N*-acetylneuraminosyl(2→3′)lactit[$^3$H]ol.
[c] NL(2→3′), α-D-*N*-acetylneuraminosyl(2→3′)lactose.
[d] n.d., not determined.

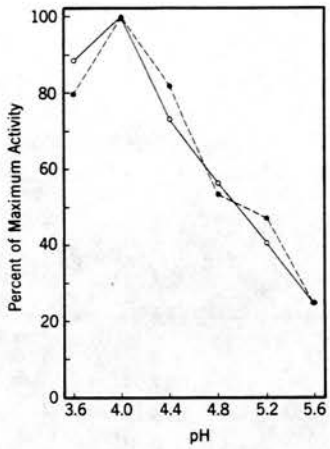


FIG. 1. pH vs activity curves of human leukocyte neuraminidase. The enzyme activity was measured by the release of lactit[$^3$H]ol (○–○) from NL[$^3$H]ol and by the release of *N*-acetylneuraminic acid (●--●) from NL(2 → 3′), respectively. The pH values shown were obtained with sodium acetate–acetic acid buffers (final concentration, 75 mM).

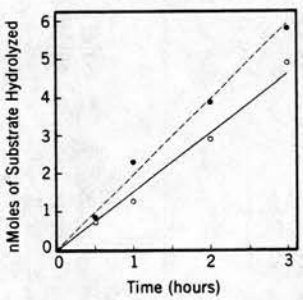


FIG. 2. Progress curve of human leukocyte neuraminidase. The incubations were conducted at pH 4.0 for the lengths of time indicated, using NL[$^3$H]ol (○–○) and NL(2 → 3′) (●--●) as the substrates.

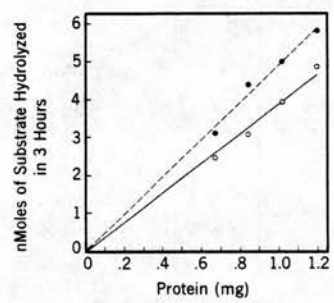


FIG. 3. Effect of protein concentration on the rate of hydrolysis. The incubations were done at pH 4.0 for 3 hr, using NL[$^3$H]ol (○–○) and NL(2 → 3′) (●--●) as the substrates.

TABLE 3
ASSAY OF NEURAMINIDASE ACTIVITY IN PREPARATIONS OF CHICK LIVER AND RAT LIVER UTILIZING α-D-*N*-ACETYLNEURAMINOSYL(2→3′)LACTIT[$^3$H]OL AS THE SUBSTRATE

| | *N*-Acetylneuraminic acid (nmoles/hr/mg protein) | | Lactit[$^3$H]ol (nmoles/hr/mg protein) |
|---|---|---|---|
| Enzyme preparation | Direct TBA assay | TBA after isolation by ion-exchange chromatography | Isolated by ion-exchange chromatography |
| Chick-liver cytoplasmic extract | 1.73 | 1.90 | 1.53 |
| Rat-liver plasma membrane | 0.23 | 2.38 | 1.54 |

For experiments with leukocyte neuraminidase, mixed human leukocytes were isolated by dextran sedimentation (20) from heparinized blood, freshly drawn from a healthy male donor. A cell suspension (8.9 mg of protein per ml) in glass distilled water (12) was utilized for these studies, and, unless stated otherwise, the incubations were done for 3 hr at 37°C. The pH versus activity curves (Fig. 1) showed a pH optimum of 4.0 whether using this isotopic method or our previous method (12) with NL(2 → 3′) as the substrate and direct TBA assay. The enzymic activities (nanomoles hydrolyzed per milligram of protein in 3 hr) at pH 4.0 were 4.41 by the isotopic method and 4.81 by the TBA assay, respectively; these values are similar to those previously reported from our laboratory (21). The rate of hydrolysis of both substrates is linear at least for 3 hr (Fig. 2) and a linear relationship was also observed between protein concentration and enzyme activity (Fig. 3).

Neuraminidase activity was also assayed in an aqueous suspension (1.92 mg of protein/ml) of rat liver plasma membrane (fraction $P_2$) prepared according to Touster *et al.* (22) and in the cytoplasmic extract obtained from a chick-liver homogenate (1:4 v/v) in 0.154 M KCl (23). These preparations were incubated for 3 hr at 37°C, pH 4.0, in presence of NL[$^3$H]ol and the enzyme activity was determined by direct TBA assay, by TBA assay after ion-exchange chromatography, and by measurement of the lactit[$^3$H]ol released (Table 3). The strong interference by spurious colors in the direct TBA assay was quite impressive for this preparation of plasma membrane and these results illustrate the problems and pitfalls often encountered in the assay of neuraminidase activity in animal tissues.

## DISCUSSION

Several approaches have been utilized for the measurement of neuraminidase activity by methods that avoid the TBA reaction. Radioactively labeled sialo-glycoproteins have been recently utilized for studies with bacterial and mammalian neuraminidases (24,25). In these experiments, the tritiated seven- and eight-carbon analogs of *N*-acetylneuraminic acid, released by the enzyme action, are assayed by measuring the radioactivity present in the acid-soluble fraction obtained from the incubation mixtures. However, the observation that these modified glycone moieties are released by neuraminidase action at a slower rate than intact *N*-acetylneuraminic acid from native sialoglycoproteins (26) and the possibility that proteases present in lysosomal preparations or in crude tissue extracts may cause the release of acid-soluble labeled sialoglycopeptides (26), indicate that the use of these substrates should be undertaken with some caution.

Synthetic substrates with chromogenic aglycones, such as the phenyl (27) and 3′-methoxyphenyl (28) $\alpha$-ketosides of *N*-acetylneuraminic acid have been tested successfully with purified neuraminidase preparations. However, since the Folin–Ciocalteau reagent utilized for the assay of the released phenolic aglycone also reacts with phenolic amino acids in proteins (13), these methods are not suitable for neuraminidase assay in crude tissue preparations (1). Substrates with aglycones which produce a chromophore by simple pH adjustments, such as nitrophenols and phenolphthalein, would circumvent some of these problems and hence attempts to prepare these compounds were undertaken in our laboratory[3]. Unfortunately, efforts to prepare *o*-nitrophenyl and *p*-nitrophenyl ketosides of *N*-acetylneuraminic acid by methods similar to those described by Tuppy and his associates (27,28) for the synthesis of phenyl glycosides were unsuccessful. There was apparently no reaction between *o*-nitrophenol and aceto-chloro-neuraminic acid (4,7,8,9-tetra-*O*-acetyl-*N*-acetyl-2-chloro-2-deoxy-D-neuraminic acid) while with *p*-nitrophenol although a product was isolated, the yields, under various experimental conditions, were too low ($< 5\%$) to warrant further work. Attempts to prepare the phenolphthalein ketoside of *N*-acetylneuraminic acid by a method similar to that used by Marsh and Reid (29) for the synthesis of phenolphthalein-glucuronide yielded small amounts of a compound which contained equivalent amounts of *N*-acetylneuraminic acid and phenolphthalein. However, since this compound was not hydrolyzed by bacterial neuraminidase, there is a strong possibility that it may be the $\beta$-ketoside.

A method based in the colorimetric assay of the lactose released by

[3] Bhavanandan, V. P., and Carubelli, R., unpublished observations.

the action of neuraminidase on neuramin-lactose was developed by Holmquist (30). A drawback of this method is that other sugars interfere with the determination of the lactose released by neuraminidase, while in the method described in this paper the radioactivity measured can only originate from the aglycone moiety of the substrate.

The observation that NL[$^3$H]ol and its parent compound NL(2 → 3′) are hydrolyzed at the same rate was fully expected since both substrates have identical composition, configuration and conformation (i.e., α-D-*N*-acetyl neuraminosyl-(2 → 3′)-β-D-galactopyranosyl) at the level of the neuraminidase susceptible linkage, and the difference between their molecular weights is negligible.

The results presented here show that the method is operational and suggest that reduction of NL(2 → 6′) and of the prosthetic group of various sialoglycoproteins with tritiated borohydride should also provide suitable substrates for this assay. Glycoproteins with sialic acid-containing prosthetic groups linked by *O*-glycosidic linkage to seryl and threonyl residues would be particularly suited for this purpose since these glycoproteins release their prosthetic groups by a mechanism of β-elimination (7). For example, treatment of ovine submaxillary glycoprotein with mild alkali in the presence of NaB[$^3$H]$_4$ would yield α-D-*N*-acetylneuraminosyl-(2 → 6′)-*N*-acetylgalactosaminit[$^3$H]ol, which could substitute for NL[$^3$H]ol in the assay system described.

In addition to its usefulness for work with crude enzyme preparations, the special features of this method make it particularly valuable for the assay of neuraminidase activity in the presence of *N*-acetylneuraminic acid-utilizing enzymes, such as *N*-acetylneuraminic acid aldolase, as well as in the presence of high concentrations of free sialic acid (inhibition studies).

Although this method in its present form has some time-consuming steps, the use of substrates with higher specific radioactivity should allow a reduction in the volumes of the incubation mixtures, columns, and effluents thus shortening and simplifying the whole operation.

## ACKNOWLEDGMENTS

The authors express their appreciation to Mrs. Anna Henley for her excellent technical assistance.

This research was supported in part by USPHS Grant NS 09176.

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*Reprinted from*
*Proc. Nat. Acad. Sci. USA*
Vol. 72, No. 6, pp. 2032–2036, June 1975

# Mucopolysaccharides Associated with Nuclei of Cultured Mammalian Cells

**(mouse melanoma/nuclear membrane/sulfated polysaccharides)**

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*Communicated by Karl Meyer, March 3, 1975*

**ABSTRACT** **Mucopolysaccharides have been isolated, fractionated, and characterized from the nuclei of cultured B16 mouse melanoma cells grown in the presence of [$^3$H]-glucosamine and [$^{35}$S]sulfate. Digestion of the nuclei with DNase followed by Pronase gave a mixture of complex carbohydrates from which the mucopolysaccharides were isolated by precipitation with cetylpyridinium chloride. After fractionation by differential salt extraction and chromatography on controlled pore glass bead columns, the components were identified by chemical and enzymatic methods. The major polysaccharide components were a family of high-molecular-weight chondroitin sulfates with different degrees of sulfation; a minor component has been characterized as heparan sulfate.**

The production of mucopolysaccharides by cultured cells and the effect of virus transformation on this has been demonstrated for several cell lines (1–5). A major portion of the mucopolysaccharide synthesized by the cells can be detected in the culture media and as intercellular material, not a surprising result, since mucopolysaccharides are considered to be extracellular components. In addition, mucopolysaccharide is detectable in cell pellets, although the nature of the association of this material with the cell has not been fully established. The presence of heparan sulfate as a surface component of several cell lines has been reported (3) but it is not clear whether the heparan sulfate–protein complex is an integral part of the plasma membrane of cells or is present as a cell coat (fuzz) immobilized by ionic interaction with cell membrane proteins.

In this paper we present evidence for the presence of mucopolysaccharides associated with cell nuclei. The presence of anionic saccharides in the nucleus of mammalian cells may have considerable significance. While cell surface complex carbohydrates can be involved in determining antigenicity, intercellular recognition and adhesion, contact inhibition, and other surface-related phenomena, anionic saccharides of the nuclei are more likely to be involved in controlling nuclear and cytoplasmic events. Alternatively these components may have a common function in all membranes as structural elements maintaining organellar integrity or regulating membrane properties, such as transport of nutrients.

## MATERIALS AND METHODS

B16 mouse melanoma cell line 3 and an amelanotic clone were propagated as described previously (5). Cells utilized for the isolation of nuclei were harvested in the late-log phase of growth at a density of about $15 \times 10^6$ cells per 16 oz (0.5 liter) bottle. In experiments requiring labeling of complex carbohydrates, the cells were cultured for 48 hr prior to harvest in sulfate-free medium containing per milliliter 10 $\mu$Ci of [$^3$H]glucosamine·HCl (New England Nuclear, 7.3 Ci/mmol) and 50 $\mu$Ci of $Na_2{}^{35}SO_4$ (New England Nuclear, 738 mCi/mmol).

Cells were harvested by pouring off the media, washing the cell layer three times with serum-free medium, and treating with 0.01 M EDTA in calcium, magnesium-free phosphate-buffered saline (10 ml) at 37° for 5 min. Serum-free medium (10 ml) was added, and the cells were collected by centrifugation and washed three times with the buffered saline by resuspension and centrifugation. The viability of the cells was in the region of 85–95% as determined by trypan blue exclusion.

Nuclei were prepared by two methods: one utilized 0.05% Triton X-100 in the buffer as described by Alwine *et al.* (6); the other method was essentially as described by Sakiyama and Burge (7) except that the nuclear pellet after the first gradient centrifugation was suspended in 4 ml of 0.2 M sucrose in buffer and layered on top of the gradient, and the centrifugation was repeated. The purification of nuclei was routinely monitored by phase contrast microscopy.

Protein was determined according to Lowry *et al.* (8) with crystalline bovine serum albumin as the standard. DNA and RNA were determined by the modified Schmidt-Thannhauser method as described by Munro and Fleck (9) with calf thymus DNA and yeast RNA as standards, respectively.

5′-Nucleotidase was assayed by the method of Dewald and Touster (10) and acid phosphatase as described by Appelmans *et al.* (11). Succinic dehydrogenase was assayed by determining the reduction of cytochrome *c* (12) or of 2-*p*-iodophenyl-3-(*p*-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (13).

The isolation of the complex carbohydrate components was achieved by suspending the washed nuclei pellet in 0.01 M Tris·HCl buffer, pH 7.4, containing 0.01 M $MgCl_2$ and digesting with DNase. The digestion was carried out at 37° for a period of 24 hr with addition of 50 $\mu$g of DNase at time 0 and again after 12 hr. The digest was exhaustively dialyzed against distilled water and the nondialyzable portion was centrifuged ($2000 \times g$, 10 min) and the radioactivity in an aliquot of the supernatant was measured. The pellet was resuspended in the supernatant (pH adjusted to 8.0) and digested with Pronase for 48 hr at 40° in the presence of toluene. The digest was centrifuged and the supernate was extensively dialyzed against 0.2 M NaCl followed by distilled water.

Hyaluronic acid, chondroitin-4-sulfate, and heparin (0.5 mg each) were added as carriers to the dialyzed digest and, after the solution was made 0.04 M in $Na_2SO_4$, 2% cetylpyridinium chloride (CPC) in 0.04 M $Na_2SO_4$ was added at 1 ml/10 ml, and the solution was mixed and allowed to stand at room tem-

Abbreviations: CPC, cetylpyridinium chloride; CPG, controlled pore glass.

perature overnight. The precipitate was collected by centrifugation (10,000 × *g*, 10 min) and washed three times with 0.2% CPC in 0.04 M $Na_2SO_4$ by resuspension and centrifugation. The CPC precipitate was then fractionally extracted with 0.2, 0.4, 0.8, 1.2, 2.0 M NaCl utilizing 2 ml once and 1 ml four times for each extraction. The CPC in the extracts was removed by dialysis at 40–45° against 0.1 M NaCl followed by distilled water at 4° and the solutions were lyophilized.

Cellulose acetate electrophoresis was carried out as described earlier (5) but using pyridineacetic acid buffer, pH 3.5, at 10 mA for 20 min or 0.2 M calcium acetate, pH 7.0, at 5 mA for 3 hr. Paper electrophoresis was done on Whatman 3MM sheets in 0.5 N pyridineacetic acid buffer, pH 5.0, at 10 V/cm for 2–3 hr. Hexosamine in isotopically labeled components was determined as described earlier (5).

Sources of enzymes, isotopes, standard mucopolysaccharides, and chemicals were as previously described (5). DNase was the electrophoretically purified grade from Worthington and was free of hyaluronidase and chondroitinase activities. Testicular hyaluronidase digestion was performed in 0.1 M sodium acetate buffer, pH 5.0, and 8 mM EDTA in the presence of toluene and chloroform at 37° for 24 hr. Bacterial hyaluronidase digestion was performed in 0.1 M sodium acetate buffer, pH 5.0, at 37° for 24 hr. Chondroitinase and chondrosulfatase digestions were according to Suzuki and coworkers (14). Nitrous acid degradation was done by treating the sample in 160 μl of water with 20 μl of 3 M $NaNO_2$ and 20 μl of glacial acetic acid at room temperature for 80 min. Excess nitrite was destroyed by addition of 50 μl of 3 M glycine and after 60 min at room temperature, the product was lyophilized.

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 (15).

## RESULTS

The recoveries of protein and radioactivity in the nuclei were 5–10% of the total cellular protein and between 4 and 9% of the cell-associated $^3H$ and $^{35}S$ activity. The DNA and RNA contents of the nuclei were 398 and 59 μg/mg of protein, respectively. The nuclei had less than 1% of the 5′-nucleotidase, a plasma membrane marker enzyme, detected in the original cell homogenate. There was no detectable activity of acid phosphatase (lysosomal enzyme marker) and succinic dehydrogenase (mitochondrial enzyme marker) in the nuclear preparations, with about 0.5 mg of protein per assay. Although high concentrations of detergent are known to solubilize the outer nuclear membrane (16), the concentration employed by us did not appear to affect the morphology of this structure. Nuclei prepared by the alternative procedure did not appear as clean as those prepared with 0.05% Triton but the incorporation patterns of radioactivity into mucopolysaccharide and glycoprotein were similar for preparations obtained by the two methods.

After treatment of the nuclei with DNase and centrifugation, about 90–95% of the radioactivity ($^3H$ and $^{35}S$) sedimented with the pellet. Pronase digestion of the pellet solubilized the labeled components; between 75 and 80% of the $^3H$ and $^{35}S$ activities in the nuclei were recovered in the soluble fraction after the treatment. Thus the sulfated and nonsulfated complex saccharides of the nuclei may be mainly associated with the nuclear membrane, which has been reported to contain glycoproteins (17–19). The possibility that a minor portion of the complex carbohydrate of the nuclei is associated with lipids (glycolipids) cannot be excluded by these studies.

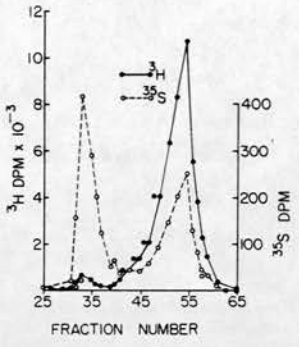


FIG. 1. Fractionation of Pronase-digested nuclear membrane pellet on a 1.5 × 115 cm controlled pore glass (CPG) 10-240 glass bead column with 0.5 M $CaCl_2$ as the eluting solvent. Fractions (4 ml) were collected and 50 μl aliquots were analyzed for radioactivity. Fractions 30–39 (Na), 40–47 (Nb), and 48–62 (Nc) were combined, dialyzed against water, and lyophilized.

Chromatography of the Pronase digest on a preparative glass bead column gave the results illustrated in Fig. 1. The majority of the sulfate radioactivity and a small percentage of the tritium label appeared in the fast-moving higher molecular weight peak (Na) and represents components derived primarily from proteoglycans. The low-molecular-weight fraction (Nc) contains the bulk of the tritium label and is predominantly glycopeptide in nature. Analytical data of these two fractions are given in Table 1.

The distribution of $^3H$ and $^{35}S$ radioactivity in the different

TABLE 1. *Analytical data of various $^3H$- and $^{35}S$-labeled macromolecular fractions from nuclei**

| Fraction | Mol. wt. | $\frac{^{35}S}{^3H}$ | GlcN | GalN | Chondroitinase AC† | |
|---|---|---|---|---|---|---|
| | | | | | $^3H$ | $^{35}S$ |
| Na | 35,000+ | — | 46.0 | 54.0 | 64.3 | 89.5 |
| Nc | ~15,000 | — | 88.3 | 11.7 | ND | ND |
| 4a | 35,000+ | 4.3 | 4.9 | 95.1 | 81.8 | 92.0 |
| 4c | 10,000 | 1.4 | 60.5 | 39.5 | ND | ND |
| 8a | 35,000+ | 9.3 | 0 | 100 | 100 | 100 |
| 8b | ~15,000 | 6.9 | 48.6 | 51.4 | 56.0 | 60.0 |
| 12a | 35,000+ | 24.7 | ND | ND | 85.4 | 86.2 |
| 12b | ~15,000 | 8.0 | 18.8 | 81.2 | ND | ND |

ND is not determined.

* Summary of analytical data for the different fractions described in the *text*. The molecular weight values are based on the behavior of the labeled components relative to standard mucopolysaccharides during chromatography on CPG columns. The amino sugar values are as percentages of tritium label found for glucosamine and galactosamine.

† Percentage radioactivity eluting in the low-molecular-weight region on a CPG column after incubation with chondroitinase AC.

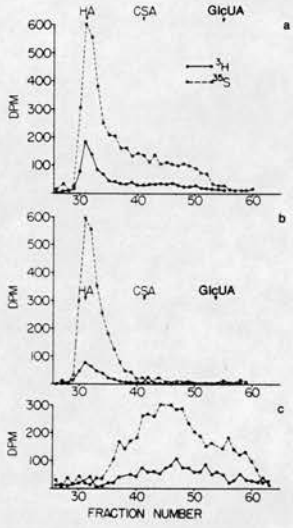


FIG. 2. (a) Fractionation of the 0.8 M NaCl extract of CPC-precipitable material from Pronase-digested nuclear membrane on a 0.9 × 95 cm column of CPG 10-240 glass beads. Fractions (1 ml) were collected and 50 μl was analyzed for radioactivity. The peak elution position of marker saccharides (HA, vitreous humor hyaluronic acid; CSA, porcine rib cartilage chondroitin-4-sulfate; and GlcUA, glucuronic acid) are indicated by the arrows. Fractions 29–35 and 36–55 were separately combined as peaks 8a and 8b, respectively. Figures (b) and (c) are elution patterns of peaks 8a and 8b after rechromatography on the same column; 700 μl of 1 ml fractions were analyzed.

CPC fractions is given in Table 2. Over 90% of $^{35}S$ and about 5% of $^3H$ activity are in salt extracts of molarity greater than 0.4 M and constitute the mucopolysaccharide content of nuclei. The CPC supernatant and the washings of the precipitate contain about 90% of the $^3H$ activity and represent glycopeptide components. A further 3% of the $^3H$ activity present in the 0.2 M NaCl eluate is a sialoglycopeptide comparable to that isolated from the culture media of the cells (5). Characterization of these glycopeptides will be described in a subsequent report.

Chromatography of the 0.8 N NaCl fraction which contained about $^2/_3$ of the sulfated polysaccharide associated with the nuclei is illustrated in Fig. 2a. Rechromatography of Fractions 8a and 8b gave the patterns shown in Fig. 2b and c. The hexosamine contents of these fractions are given in Table 1.

TABLE 2. *CPC precipitation and salt elution of $^3H$ and $^{35}S$ radioactivity in Pronase digest of nuclei**

| | CPC-soluble† | Molarity of NaCl for eluate | | | | |
|---|---|---|---|---|---|---|
| | | 0.2 | 0.4 | 0.8 | 1.2 | 2.0 |
| $^3H$ | 1548.3 (91.6) | 58.3 (3.4) | 28.8 (1.7) | 28.3 (1.7) | 5.9 (0.3) | 2.5 (0.15) |
| $^{35}S$ | 5.3 (3.5) | 3.1 (2.1) | 16.9 (11.3) | 96.6 (64.4) | 21.0 (14.0) | 1.0 (0.67) |

* Values are expressed in dpm × $10^{-3}$; the numbers within parentheses are the percentages. Results of a duplicate experiment were identical. Recovery of radioactivity was essentially quantitative.

† Material present in the CPC supernate combined with washes of the original CPC precipitate.

*Fraction 8a.* The chromatographic elution profiles of 8a after treatment with testicular and bacterial hyaluronidases and with chondroitinase AC are shown in Fig. 3. Control digestion of 8a with buffer alone and rechromatography gave a profile identical to that in Fig. 2b. The presence of galactosamine as the only hexosamine in this fraction and its complete susceptibility to testicular hyaluronidase and chondroitinase AC suggests that it consists of chondroitin-4 and/or 6-sulfate. This fraction was also susceptible to bacterial hyaluronidase but the fragments obtained were larger than tetrasaccharides, implying that the degree of sulfation is greater than 50%, since a polysaccharide with significantly less sulfation would give rise to di- and tetrasaccharides. The electrophoretic mobility of this component, in the two buffer systems tested, was consistent with that of a chondroitin sulfate having 0.5–1.0 sulfate residues per disaccharide unit. Chondroitinase AC and chondroitinase AC plus chondro-4-sulfatase digestion of this fraction followed by paper chromatography indicated that the sulfate ester group is predominantly in the 4-position of the galactosamine moiety.

After mild acid hydrolysis (0.04 N HCl, 100°, 90 min) of the 8a fraction, about 45% of the $^{35}S$ radioactivity migrated with inorganic sulfate on paper electrophoresis as well as on a Bio-Gel P2 column. It was also evident from these experiments that the polymer underwent considerable degradation. Both cartilage chondroitin-4-sulfate and hyaluronic acid treated in the same manner are degraded to fragments ranging from tetra-(major) to octa-saccharides, an observation not previously recorded. Previous findings from this laboratory (20) have established that about 35% of $^{35}SO_4^{2-}$ was released from chondroitin-4-sulfate on hydrolysis with dilute acid under conditions comparable to those used in the present study.

The possible presence of *N*-sulfate residues in this fraction was considered even though the results of the chondroitinase AC and chondro-4-sulfatase digestions were not consistent with this. Treatment of the 8a fraction with nitrous acid, a reagent that causes deaminative cleavage of mucopolysaccharides containing sulfated amino groups (21), followed by chromatography on a CPG 10-240 column gave a profile identical to that in Fig. 2b, indicating that there were no such residues in this fraction. Gel filtration of Fraction 8a on the CPG 10–240 column indicated that this component has a molecular weight of 35,000 or greater, since it elutes in the void volume coincident with vitreous humor hyaluronic acid of molecular weight 35,000, whereas cartilage chondroitin-4-sulfate of molecular weight 15,000 elutes differently (Fig. 2). A high-molecular-weight chondroitin fraction isolated from the culture media of the same cells (B16 mouse melanoma) has been estimated to have a molecular weight of 97,000 ± 8,800 by ultracentrifugation. (Bhavanandan and Davidson, unpublished results.) In order to determine whether the high molecular weight of Fraction 8a was due to incomplete Pronase digestion resulting in proteoglycan fragments rather than polysaccharide chains, we digested Fraction 8a with Pronase (two additions of 250 μg each at time 0 and 24 hr) for a total period of 72 hr and rechromatographed the product

on a CPG 10-240 column. The elution profile was identical to that of the untreated sample (Fig. 2b), indicating that the high-molecular-weight nature of this fraction is probably not due to incomplete Pronase digestion. Soluble pig cartilage proteoglycan is completely degraded to polysaccharide chains of molecular weight about 15,000 on digestion with Pronase (22).

*Fraction 8b.* The labeled hexosamine content of this fraction was 51% galactosamine and 49% glucosamine (Table 1). Chromatography on CPG 10-240 gave the heterogenous elution profile shown in Fig. 2c; high-molecular-weight material is absent and the major portion of the fraction eluted in the 15,000–10,000 range. On treatment with chondroitinase AC and rechromatography on the same column, about 60% of the $^{35}S$ activity eluted in the low-molecular-weight region. Mild acid hydrolysis followed by paper electrophoresis of this fraction also gave results similar to those obtained with Fraction 8a; about 52% of $^{35}S$ radioactivity migrated with inorganic sulfate. Nitrous acid treatment of this fraction followed by chromatography on the CPG 10-240 column showed that 57.5% of $^{35}S$ activity eluted in the low-molecular-weight region. These results suggest that this fraction is a mixture of heparan sulfate and chondroitin (4 or 6) sulfate of molecular weight similar to that of the cartilage polysaccharide.

Chromatography of the 0.4 M and 1.2 M NaCl eluates on the CPG 10-240 column gave the profiles shown in Fig. 4. Hexosamine and chondroitinase susceptibility data on Fractions 4a, 4c, 12a, and 12b are summarized in Table 1. Fractions 4a and 12a are high-molecular-weight chondroitin sulfate components, apparently the undersulfated and more fully sulfated homologs of Fraction 8a (see $^{35}S/^{3}H$ in Table 1). Fraction 4c contains lower molecular weight material composed of both amino sugars and sialic acid and is presumably sialoglycopeptide similar to that present in the 0.2 M NaCl eluate. There was insufficient material for complete characterization.

## DISCUSSION

This study provides direct biochemical evidence for the association of anionic polysaccharides with mammalian cell nuclei. In the system investigated, a family of high-molecular-weight chondroitin-4-sulfates were the major nuclear mucopolysaccharides. The other mucopolysaccharides identified were heparan sulfate and a chondroitin sulfate of lower molecular weight.

The presence of glycoproteins in cell nuclei has been previously reported by several workers (17–19) and is confirmed by our results. Based entirely on the tritium incorporation data (Table 2), it would appear that mucopolysaccharides are a minor component compared to glycoproteins; however, definitive information on the carbohydrate composition of nuclei must await mass analytical data. It is also essential to establish whether the association of mucopolysaccharides with the nuclei is a feature common to all mammalian cells.

The physical properties and chemical architecture of the anionic polysaccharides with heterogeneity of size and distribution of functional groups ($O$-$SO_4$; $N$-$SO_4$; COOH) make them excellent candidates for a range of regulatory functions, including control of cationic environment at the nuclear membrane, transport selectivity into the nuclei, regulation of nuclear enzyme activity, and control of template activity in

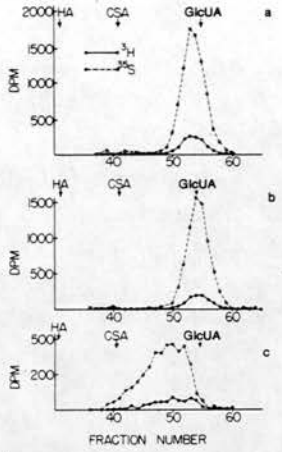


FIG. 3. Chromatography of Fraction 8a on a 0.9 × 95 cm glass bead column after digestion with testicular hyaluronidase (upper), chondroitinase AC (middle), or bacterial hyaluronidase (lower). Fractions (1 ml) were collected and 700 μl analyzed for radioactivity. Abbreviations are as in Fig. 2.

chromatin. Several recent reports have suggested involvement of anionic polysaccharides in nuclear functions. Anionic polysaccharides such as gum tragacanth (23) and dextran sulfate (24) have been shown to inhibit mitosis *in vitro* and *in vivo*. Heparin has been used to prepare nuclear membranes from nuclei, apparently because of its ability to interact with histones, thus solubilizing chromatin (25). Cook and Aikawa (26) have demonstrated the activation of endogenous DNA polymerase of rat liver nuclei by heparin. Another possible function of acidic polysaccharides in regulating DNA synthesis is indicated by the studies of Robbins *et al.* (27). Kinoshita has presented evidence (28) for a heparin-like polysaccharide which is released within the cytoplasm during cleavage of sea

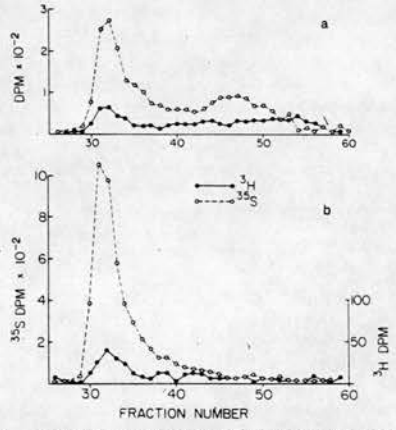


FIG. 4. Chromatography of 0.4 M (upper) and 1.2 M (lower) fractions on the CPG column. Fractions (1 ml) were collected and 50 μl was analyzed for radioactivity. Fractions 29–35 (4a) and 44–55 (4c) of the upper figure and Fractions 29–35 (12a) and 36–55 (12b) of the lower figure were combined, dialyzed, and lyophilized.

urchin embryos and is suspected to induce changes in embryonic nuclei, such as loosening of chromatin and the initiation of RNA synthesis.

The accumulation of dermatan sulfate and/or heparan sulfate in lysosomes of fibroblasts from patients with mucopolysaccharidoses is well established (29). There is a strong possibility that the lysosomal pool of polysaccharides may in part arise from nuclei by disruption of the nuclear membrane during the mitotic phase of the cell cycle. Information on this is expected from studies on the relationship between the nuclear polysaccharides and turnover of polysaccharides in the lysosomes during the cell cycle.

We are grateful to Mr. Tom Wright for technical assistance and Mr. Mord Moore for hexosamine analysis. This work was supported in part by Grant CA15483 from the U.S. Public Health Service.

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[CANCER RESEARCH 36, 424–431, February 1976]

# Anionic Polysaccharide Production and Tyrosinase Activation in Cultured Human Melanoma Cells[1]

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## SUMMARY

A human melanoma cell line established in our laboratory was characterized in terms of tyrosinase activity and anionic polysaccharide production. Tyrosinase levels were diluted during the growth phase and increased after the cell culture became confluent. The anionic polysaccharides produced included hyaluronic acid, heparitin sulfate, and a high-molecular-weight chondroitin 4-sulfate. In contrast, a primary culture of human melanocytes derived from embryonic iris produced much greater amounts of hyaluronic acid, about 30-fold less heparitin sulfate, and a mixture of chondroitin 4-sulfate and dermatan sulfate. Saccharides secreted into the culture medium were generally identical to those remaining cell associated except for the melanoma heparitin sulfate, wherein the latter fraction appeared to be of lower molecular weight.

## INTRODUCTION

Human melanoma cells bear a common antigen, cross-reactive between different patients, and unique antigen(s) that are specific for each individual tumor (4, 9, 11, 12). Some preliminary attempts have been made to isolate and characterize membrane-associated melanoma antigens. Partially purified, soluble protein fractions have been obtained from homogenized tumors by gel filtration (6) and electrophoresis (12). It is probable that membrane-associated glycoproteins contribute to the immunogenicity of human melanoma cells.

Melanoma cell cultures provide an opportunity to conduct studies on the characterization of membrane-associated glycoproteins upon a consistent cell population. Human melanoma lines have been established in a number of laboratories (7, 14) including our own.

The present study is a description of some of the biological properties of one of our lines (HM7) and the biochemical characterization of the pronase- and alkali-stable cell-associated and extracellular glycosaminoglycans and glycoproteins labeled by inclusion of tritiated glucosamine and $^{35}SO_4^{2-}$ in the culture media. The components will be compared with those produced by a population of normal melanocytes derived from an embryonic human iris explant. A preliminary report has been presented (2).

[1] This work was supported in part by Grants CA15483 and CA11097-06 from the NIH, USPHS.

[2] Career Development Award, 7-K4-CA38,809.

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Received August 11, 1975; accepted October 20, 1975.

## MATERIALS AND METHODS

**Establishment of Melanoma Cell Culture Line.** The human melanoma cell line, HM7, was obtained from an excisional biopsy of an axillary nodal metastasis in April, 1974. Chips (1 × 1 mm) of tumors were partially dried onto the surface of a plastic culture flask, incubated with medium for 1 week, and then refed 3 times each week. After a confluent monolayer was obtained, the cells were suspended with 0.04% EDTA and passaged. They have since been passaged at about 2-week intervals in 16-oz prescription bottles.

**Establishment of Human Iris Cultures.** Human iris melanocytes were obtained from a therapeutically aborted fetus utilizing methodology analogous to that described earlier (19). After aseptic excision, the 2 irises were cultured as explants for 49 hr in 7 ml of complete media containing [$^3$H]glucosamine and $Na_2{}^{35}SO_4$, 20 $\mu$Ci/ml each, in a 60-mm plastic Petri dish (Falcon Plastics, Oxnard, Calif.). At the end of this time the media were decanted, the cells were harvested by centrifugation, and each fraction was prepared for analysis. Cell counts on the iris explant were not performed due primarily to technical difficulties. In view of the significant saccharide synthesis observed, it seems probable that the bulk of the explant cell population was viable under the conditions of the experiment, although significant cell death cannot be ruled out.

**Propagation of Cultures.** HM7 cells were routinely propagated in MEM[4] with Earle's balanced salt solution supplemented with heated (56° for 30 min) fetal calf serum to 10% final concentration, sodium pyruvate, nonessential amino acids, 2× vitamins, and 100 units penicillin G per ml and 100 $\mu$g streptomycin sulfate per ml. All components, with the exception of the antibiotics and the fetal calf serum, were obtained from Grand Island Biological Co., Grand Island, N. Y. Cells were incubated at 36° in a humidified 5% $CO_2$:95% air atmosphere and were propagated in 16-oz glass prescription bottles (Brockway Glass Co., Inc., Brockway, Pa.). The cells became confluent at approximately $6 \times 10^6$ cells/ bottle and were routinely subcultured to approximately $1 \times 10^6$ cells/bottle by suspension with 0.02% ethylene glycol bis($\beta$-aminoethyl ether)-*N,N'*-tetraacetic acid in calcium- and magnesium-free phosphate-buffered saline, 0.15 M $Na^+$:0.005 M $K^+$:0.14 M $Cl^-$:0.009 M $PO_4$, pH 7.2. Cultures were routinely tested for bacterial contamination by the use of thioglycolate and tryptose phosphate broth and for yeast

[4] The abbreviations used are: MEM, Eagle's minimum essential medium; CPC, cetyl pyridinium chloride; HS, heparitin sulfate; HA, hyaluronic acid; CPG, controlled porous glass; H.I.M., human iris melanocytes; C4S, chondroitin 4-sulfate.

and mold contamination with Sabouraud's medium. No contamination was observed. The possible presence of mycoplasma was not assessed in the cells used for labeling studies although other lines carried in our laboratory have been routinely assayed and found to be free of contamination.

**Examination of Karyotype.** Petri dishes (60 mm) were seeded with $10^6$ dye-excluding cells 3 to 5 days before karyotyping experiments. The cultures were treated with colchicine, 0.25 μg/ml (Calbiochem, San Diego, Calif.), for 2 hr, resuspended with 0.25% trypsin in phosphate-buffered saline, and washed twice with MEM. The cell pellet was resuspended in a hypotonic (0.6% sodium citrate:0.06 M KCl) solution, incubated for 30 min at 37°, and then washed twice with MEM. The cell pellet was fixed with methanol:acetic acid (3:1, v/v), refrigerated for 20 min, and washed twice in the fixative. The pellet was broken up with a Pasteur pipet and 2 to 3 drops were placed on a microscope slide. The fixative was evaporated on a warm hotplate and the preparation was stained with 2% Giemsa.

**Implantation of Cells into Nude Mice.** Human melanoma cells grown to confluency in 16-oz prescription bottles were suspended with 0.1% EDTA, washed, and counted, and $5 \times 10^6$ trypan blue-excluding cells were inoculated s.c. into the backs of 10 CRL-HO nude mice (Drug Research and Development Branch, National Cancer Institute, Bethesda, Md.). The mice were housed under ordinary ambient laboratory conditions but with tetracycline added to the drinking water.

**Cell Labeling and Sizing.** Two confluent bottles (16 oz) of human melanoma cells were fed with 25 ml of complete media containing [$^3$H]glucosamine, 5 μCi/ml, and $Na_2{}^{35}SO_4$, 25 μCi/ml, for 48 hr and then were prepared for analysis. To increase the labeling of the cellular components, confluent cultures were labeled for 48 hr in media containing no inorganic sulfate and one-third the glucose concentration of complete media. Essentially similar results were obtained using either labeling procedure. Separate experiments conducted with early- and late-passage logarithmically growing cells gave essentially identical results. No attempt was made to evaluate specific saccharide synthesis as a function of the cell cycle, although other results suggest that the saccharide pattern seen is independent of cell density (J. Banks and E. A. Davidson, unpublished results).

Cell sizing was done utilizing a Model B Coulter counter. Ragweed pollen and previously calibrated B16C3 mouse melanoma cells grown in parallel were used for calibration.

**Analysis of Anionic Polysaccharides.** Saccharide fractions were isolated from culture medium and cell-associated material by fractional salt elution from CPC precipitates of pronase-digested and alkali-treated samples as described by Satoh *et al.* (19).

Liquid chromatography in 0.5 M $CaCl_2$ utilizing previously calibrated 10-240 80/120 beads (Electro-Nucleonics, Inc., Fairfield, N. J.) was done as described previously (19) and in the indicated figures. Recoveries for these runs were greater than 90% and standards were always included.

Amino sugar analyses of 5,000 to 20,000 $^3$H dpm of the labeled samples were done by hydrolysis in 6 N HCl for 24 hr at 100° in a vacuum. Recovery was 70 to 80% for polysaccharide samples with the losses occurring during hydrolysis. The hydrolysates were dried in a vacuum over $P_2O_5$ and NaOH and were then chromatographed on a Beckman 120C amino acid analyzer using a stream-splitting attachment.

For liquid scintillation counting of samples, the volume of the sample was brought to 1 ml, and 10 ml of a scintillation cocktail (1) were added. The counting efficiency of tritium in this system was 15 to 16% and, of $^{35}$S, was 46%, utilizing an Intertechnique SL36 liquid scintillation spectrometer. Crossover of $^{35}$S counts into $^3$H window was usually 13.5%, while $^3$H count crossover into the $^{35}$S window was negligible and was not corrected. No additional quenching was observed with any of the solvents at the concentration used for the experiments. For stream-splitting analyses, 4-ml fractions were collected and counted utilizing 10 ml of 3a40 complete scintillation cocktail (Research Products International Corp., Elk Grove Village, Ill., 60007).

D-Glucosamine-6-[$^3$H]hydrochloride, 7.3 Ci/mmole, and carrier-free $Na_2{}^{35}SO_4$, 80 to 800 mCi/mmole, were obtained from New England Nuclear, Boston, Mass.

The enzymes and other components were obtained from previously described sources (19) with the exception of leech hyaluronidase, which was obtained from Biotrics, Arlington, Mass. Heparitinase and heparinase were isolated from an adapted *Flavobacterium heparinum* strain obtained from Dr. A. Linker. The enzyme purifications were followed through the initial hydroxylapatite column elution (15). At this point, contamination of the heparinase and heparitinase peaks by glucuronidase and chondroitinase was considered low enough to pool appropriate tubes for enzyme assays.

Conditions for the enzyme assays were 0.25 unit chondroitinase ACII or ABC, 24 hr at 37° in 0.1 M phosphate buffer, pH 7.8, or in 1 to 5 diluted enriched Tris buffer (18). The total volume for the enzyme digest was 500 μl; 250 μg of a chondroitin 4:6 chondroitin sulfate mixture was completely hydrolyzed when run in parallel with the sample that contained less than 15,000 $^3$H dpm. These enzymes had no significant heparitinase activity. For the heparitinase assays, the samples with HS or heparan sulfate were digested for 24 hr in 0.1 M sodium acetate buffer, pH 7, containing 1 mM calcium acetate. The temperatures for incubation were 30° for heparinase and 43° for heparitinase. Contaminating chondroitinase accounted for less than 10% of the activity. Samples for leech hyaluronidase digestion were incubated in McIlvaine's standard buffer at 37° for 6 hr in a 400-μl volume. Leech hyaluronidase, 125 μg, was added every hr for the 1st 3 hr to ensure complete digestion of HA.

For paper chromatography of chondroitinase ACII- and/or ABC-digested samples, the volume of the enzyme digest was 50 μl in enriched Tris buffer as described by Saito *et al.* (18). Chondroitin 4-sulfate and chondroitin 6-sulfate standards were digested with the enzymes to detect any sulfatase activity. No significant activity was found. Radioactive chromatograms were cut into 1-inch strips after drying and added to a counting vial containing 2 ml of deionized $H_2O$. After overnight shaking, 15 ml of a scintillation cocktail were added, and after thorough mixing the samples were counted. Elution of applied $^3$H and $^{35}$S dpm under these conditions was greater than 90%.

**Tyrosinase Assay.** Tyrosinase activity *in situ* of living cells in culture was determined by a recent modification (16) of the Pomerantz method (17). Briefly, tritiated L-tyrosine (New England Nuclear) was added to the cultures at a final concentration of 1 μCi/ml. After 24 hr, a 200-μl aliquot of supernatant was removed. The aliquot was adsorbed with Norit A and the soluble fraction was counted in Aquasol (New England Nuclear) in a liquid scintillation spectrometer.

Cellulose acetate electrophoresis was carried out in 0.2 M calcium acetate, pH 7.0, at 5 ma for 3 hr or in pyridine formic acid buffer, pH 3.0, at 10 ma for 30 min.

## RESULTS

**Characteristics of HM7 Cell Monolayers.** The doubling time of the HM7 cells after 20 passages decreased to approximately 40 hr from a mean value of 84 hr after 5 passages but has not changed significantly in several recent experiments. This is somewhat longer than the average value reported by Liao *et al.* (14) for several human melanoma lines, but, since our growth medium and substratum differ, direct comparison cannot be made. Viability, as measured by trypan blue exclusion, was better than 80%. The cells of the HM7 line were considerably variable in size. Relatively small cells with scanty cytoplasm predominated, but there were also occasional cells 6 to 10 times the diameter of the smallest cells. The large cells were amelanotic and contained single nuclei. The observed morphological heterogeneity may in part derive from the fact that this line has not been cloned. However, we believe that some of the differences noted may be related to the maturational state of the cells.

With continued growth and the attainment of confluency, the HM7 monolayer developed multilayered foci characteristic of transformed cell lines. Cells in these foci sometimes presented amber-brown pigmented granules, similar to those described in other melanotic human melanoma cell lines (14).

A frequency distribution of cell volume was obtained with the Coulter counter and showed a mean cell volume of 1980 cu μm (Chart 1).

**Implantation of HM7 Cells into Nude Mice.** Nude mice inoculated with HM7 cells developed small (2 to 3 mm), plaque-like, s.c. nonpigmented nodules. The nodules were not elevated and did not spread beyond the injection sites. Microscopic sections of the nodules demonstrated pleomorphic amelanotic cells with conspicuous nucleoli and nuclear cytoplasmic evaginations. Death of all animals occurred as a consequence of intercurrent bacterial infection.

**Relationship of Cell Population Density and Tyrosinase Activity.** The production of $^3H_2O$ is a measure of the *in situ* functional activity of tyrosinase (16). During the replicative phase of culture growth, the tyrosinase activity progressively decreased each day until the 9th culture day, when the monolayer became confluent and tyrosinase levels were minimal (Chart 2). With the attainment of confluency, tyrosinase activity increased until it was restored to its former level by the end of the experiment at 17 days.

**Evaluation of HM7 Karyotype.** An enumeration of the numbers of chromosomes in spread metaphases showed a very broad distribution of values. However, about two-thirds of the metaphase cells contained 40 to 60 chromosomes. Although the chromosomes could not be paired in consistent patterns, most of those studied were metacentric, a characteristic of human chromosomes.

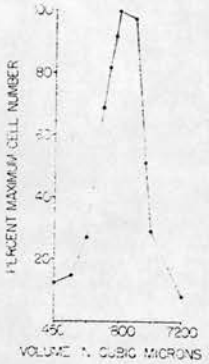


Chart 1. Cell volume measurement. Cells in the logarithmic phase of growth were harvested with phosphate buffered saline:0.02% ethylene glycol bis(β-amino ether)-*N,N'*-tetraacetic acid solution and resuspended in Isoton (Coulter Electronics, Inc., Hialeah, Fla.) at approximately 1 × $10^5$ cells/ml. The same mean volume was obtained when the experiment was repeated several passages later.

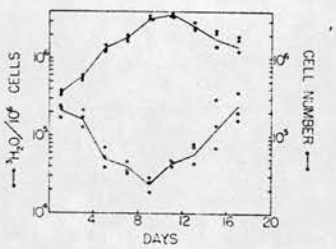


Chart 2. Growth curve of cells used for tyrosinase assay and results of tyrosinase assay.

**Saccharide Fractionation and Characterization.** Summary data of $^3H$ and $^{35}S$ incorporation at the final stages in the standard workup procedure (19) are shown in Table 1. From 6.6 to 26% of the incorporated $^3H$ and $^{35}S$ is found in CPC-precipitable material (Table 2). After dialysis to remove residual CPC, the nonprecipitable human melanoma media fraction was examined by high voltage electrophoresis for the presence of keratan sulfate; none was found. The $^3H$-labeled material migrated only slightly from the origin, whereas the $^{35}S$-labeled material little of which remained, migrated completely from the strip in a manner identical to that of inorganic sulfate.

**The 0.4 M NaCl Eluate.** Fractionation of the 0.4 M NaCl eluates from the media on CPG-10 240 80/120 glass beads resulted in the patterns shown in Chart 3. Incubation of the samples from the melanoma or the iris culture with bacterial or leech hyaluronidase resulted in complete degradation of the media saccharides and degradation of about 90% of the material in the cell-associated fraction. These results indicate that both the cell and media fraction contained HA almost exclusively. The cell-associated fraction from the

Table 1
*Incorporation of precursor label into macromolecular products*

| Fraction | Stage[a] | $^3H$ dpm/$10^3$ cells | $^{35}S$ dpm/$10^3$ cells |
|---|---|---|---|
| HM7 media | CPC supernatant | 1657 | 367 |
| HM7 cell associated | CPC supernatant | 270 | 27 |
| | | $^3H$ dpm × $10^{-3}$ | $^{35}S$ dpm × $10^{-3}$ |
| HM7 media | CPC supernatant | 11,684.6 | 2,585.4 |
| HM7 cell associated | CPC supernatant | 1,906.6 | 189.6 |
| H.I.M media | CPC supernatant | 3,322 | |
| H.I.M cell associated | CPC supernatant | 1,300 | |

[a] Stage refers to labeled nondialyzable material remaining in solution after addition of CPC to 0.2% CPC (final concentration) in 3 mM NaCl and addition of carrier mucopolysaccharides (19) and celite. This material appears to be mainly glycopeptide in nature.

Table 2
*Summary of CPC elution results*

Results for the elution values presented in Part A are in dpm/$10^3$ cells. For Parts B and C the values presented are expressed as dpm × $10^{-3}$.

| | Salt eluate fraction | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.4 | | 0.8 | | 1.2 | | 2.0 | | Totals | | % precipitated[a] | |
| | $^3H$ | $^{35}S$ | $^3H$ | $^{35}S$ | $^3H$ | $^{35}S$ | $^3H$ | $^{35}S$ | $^3H$ | $^{35}S$ | $^3H$ | $^{35}S$ |
| A. HM7[b] media | 22.8 | 1.5 | 26.0 | 18.6 | 30.6 | 25.5 | 36.9 | 28.6 | 116.3 | 74.2 | 6.6 | 16.8 |
| HM7 cell associated | 16.2 | .2 | 27.2 | 3.6 | 11.7 | 4.8 | 3.2 | 1.1 | 58.3 | 9.7 | 17.7 | 26.0 |
| B. H.I.M. media | 656.7 | .17 | 47.5 | 1.4 | 45.8 | 3.6 | 99.4 | 12.5 | 849.4 | 17.7 | 20.4 | |
| H.I.M. cell associated | 111.1 | 1.9 | 25.5 | 1.6 | 13.0 | .9 | 24.7 | 5.3 | 174.3 | 9.7 | 11.8 | |
| C. HM7 media | 160.9 | 10.3 | 183.6 | 131.4 | 215.9 | 179.6 | 260.9 | 201.7 | 821.3 | 523.0 | 6.6 | 16.8 |
| Cell associated | 114.4 | 1.4 | 191.5 | 25.7 | 82.7 | 33.8 | 22.3 | 7.9 | 410.9 | 68.8 | 17.7 | 26.6 |

[a] $^3H$ and $^{35}S$ dpm in the CPC-precipitated fraction divided by the sum of the non-CPC-precipitated and the CPC-precipitated dpm.
[b] The number of HM7 cells used was $7.05 \times 10^6$ in two 16-oz bottles.

human melanoma was resolved by preparative chromatography on a glass bead column. The lower-molecular-weight fraction contained both glucosamine (80%) and galactosamine (20%) as well as sialic acid. The latter component contained somewhat more than one-half of the radioactivity in this fraction and indicates that the major if not exclusive materials present are sialoglycopeptides. These results were confirmed by electrophoresis and are summarized in Table 3.

**The 0.8 M NaCl Eluate.** Fractionation of the 0.8 M NaCl eluates on CPG-10 240 80/120 glass beads resulted in the patterns shown in Charts 4 and 5. Incubation of the human melanoma media fraction with heparitinase resulted in digestion of 80% of the $^3H$ dpm and 95% of the $^{35}S$ dpm (Chart 4). The remaining 20% of the $^3H$ dpm was a 1:1 mixture of chondroitin 4:chondroitin 6 sulfate and HA based on amino sugar content, cellulose acetate electrophoresis, and susceptibility to leech hyaluronidase and chondroitinase ACII. Following digestion of the media fraction with chondroitinase ACII, 9% of the $^3H$ and 7% of the $^{35}S$ radioactivity migrated in the disaccharide region after chromatography on a Bio-Gel P2 column. The excluded peak contained glucosamine exclusively and had a mobility on the CPG column somewhat slower than the C4S standard. This change in apparent molecular weight may arise from contamination of the enzyme preparation or the existence of a hybrid molecule containing both HS and C4S. The bulk of the human iris media fraction contained a mixture of HA and chondroitin 4:chondroitin 6 sulfate as well as small amounts of HS and dermatan sulfate (Chart 5). The composition of the cell-associated and media fractions for both the melanoma and iris were very similar. However, the melanoma heparan sulfate from the medium appeared to have a higher molecular weight than that which was cell associated. Results are summarized in Table 4.

**The 1.2 M NaCl Eluate.** Fractionation of the melanoma 1.2 M NaCl eluate on a CPG-10 240 80/120 glass bead column resulted in the patterns shown in Chart 6. Incubation of the melanoma fraction with chondroitinase ACII resulted in hydrolysis of approximately 58% of the $^3H$ dpm and 87% of the $^{35}S$ dpm; incubation with heparitinase resulted in hydrolysis of 52% of the $^3H$ dpm. These results agree well with the amino sugar analyses indicating a 1:1 mixture of glucosamine and galactosamine and were confirmed by cellulose acetate electrophoresis. The human iris sample differed in that 90% of the material was a chondroitin sulfate, presumably chondroitin 4-sulfate. The presence of a small amount of dermatan sulfate could not be ruled out since dermatan sulfate was present in both the 0.8 and 2.0 M NaCl eluates. The identification of the glucosamine-containing compo-

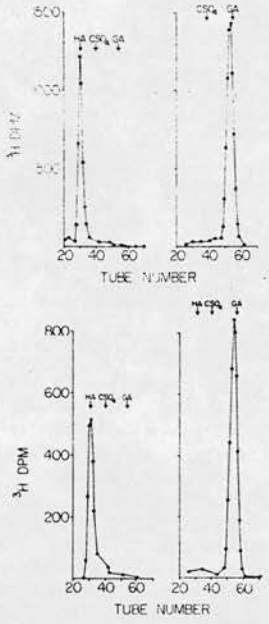


Chart 3. Results of the chromatography on CPG-10 240 80/120 glass beads of the 0.4 M NaCl eluates of the human melanoma (*upper*) and iris media (*lower*) fractions. *Left*, material before bacterial hyaluronidase digestion. *Right*, material after bacterial hyaluronidase digestion. $CSO_4$, chondroitin 4-sulfate; *GA*, glucuronic acid.

Table 3

*Properties of 0.4 M NaCl fraction*

Chromatography of the samples was done on a 0.9 x 60 cm column of CPG-10 240 80/120 glass beads. The eluting solvent was 0.5 M $CaCl_2$ and each fraction was approximately 1 ml. The flow rate was maintained at 30 ml/hr using a pump from Buchler Instruments.

| | M.W. | % $GluNH_2$ | % $GalNH_2$ | Bacterial hyaluronidase[a] (%) |
|---|---|---|---|---|
| HM7 media | 35,000+ | 99 | | 100 |
| H.I.M. media | 35,000+ | 100 | | 100 |

[a] Susceptibility to digestion assayed by exclusion chromatography on glass beads or Bio Gel P2.

nent as HS was not confirmed due to the small number of $^3$H dpm incorporated into this sample. The cell-associated components were identical to the media fractions (Table 5).

**The 2.0 M NaCl Eluate.** Fractionation of the 2 M NaCl eluates on the CPG-10 240 80/120 glass bead column showed both the melanoma and iris to contain a single high-molecular-weight fraction. Incubation of the human melanoma media fractions with chondroitinase ACII, followed by paper chromatography (Chart 7) of the digested material, indicated the presence of chondroitin 4-sulfate only since the Δ-disaccharide-4-sulfate was obtained after enzyme hydrolysis in 88% yield. Incubation of the human iris media fraction with chondroitinase ACII resulted in 85% susceptibility by the dialysis assay. However, when incubation with the enzyme was followed by product analysis by Bio-Gel P2 or paper chromatography, it was obvious that the bulk of the material was incompletely hydrolyzed. The maximum amount of chondroitinase ACII-susceptible material was 62% by these methods. Following chondroitinase ABC incubation, however, the sample was completely digested to disaccharides; the main unsaturated disaccharide present was Δ-disaccharide-4-sulfate (18). These results agree well with the amino sugar analyses, and it may be concluded that this fraction contains both chondroitin 4-sulfate and dermatan sulfate (Table 6).

A summary of the incorporation data for the melanoma and iris melanocyte anionic saccharides and their relative amounts is given in Table 7.

## DISCUSSION

The HM7 line was composed of a morphologically heterogeneous population of cells including a preponderance of small amelanotic cells and occasionally much larger forms. We observed similar changes in the B16C3 melanoma cell line and produced evidence supporting the conclusion that the heterogeneity was the result of the murine B16 melanoma cells maturation and differentiation pattern (13). Although a similar interpretation for the heterogeneity of the

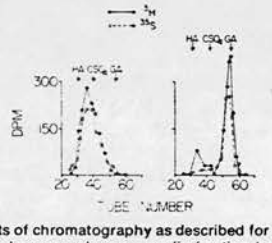


Chart 4. Results of chromatography as described for Chart 3 of the 0.8 M NaCl eluate of the human melanoma media fraction before (*left*) and after (*right*) heparitinase/heparinase digestion. $CSO_4$, chondroitin 4-sulfate; *GA*, glucuronic acid.

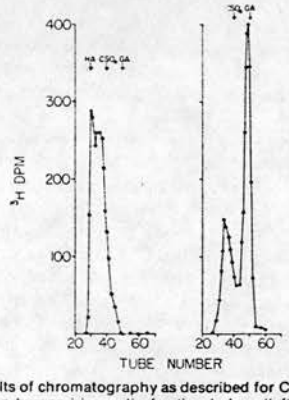


Chart 5. Results of chromatography as described for Chart 3 of the 0.8 M NaCl eluate of the human iris media fraction before (*left*) and after (*right*) bacterial hyaluronidase digestion. $CSO_4$, chondroitin 4-sulfate; *GA*, glucuronic acid.

Table 4
*Properties of 0.8 M NaCl fractions*

| | M.W. | $GluNH_2$ | $GalNH_2$ | Hyaluronidase (%) | Chondroitinase ACII (%) | Heparitinase (%) |
|---|---|---|---|---|---|---|
| HM7 media | 35,000–14,000 | 90 | 10 | 10[a] | 10 | 80 |
| H.I.M. media | 35,000–14,000 | 54 | 46 | 60[b] | 21 | 7 |

[a] After heparitinase, heparinase, and chondroitinase digestion and extensive dialysis the remaining material was subjected to leech hyaluronidase digestion, and after lyophilization the sample was chromatographed on a calibrated CPG-10 240 80/120 with marker mucopolysaccharides (19) (unlabeled). A molecular weight range of 35,000 to 14,000 daltons indicates the material was intermediate in molecular weight to the HA (M.W. 35,000) and $CSO_4$ (M.W. 14,000) markers used.
[b] Bacterial hyaluronidase susceptibility.

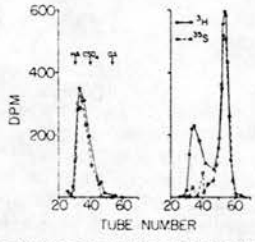


Chart 6. Results of chromatography as described for Chart 3 of the 1.2 M NaCl eluate of the human media fraction before (*left*) and after (*right*) chondroitinase AC digestion. *CSO*$_4$, chondroitin 4-sulfate; *GA*, glucuronic acid.

HM7 line is heuristically appealing, we have not yet examined this possibility.

The activity of tyrosinase in this system is dependent upon the replicative activity of the cells. This observation is similar to that which we found in B16C3 cells (J. W. Kreider, unpublished observations) but with some important differences. In the B16C3 line, tyrosinase activity was not detectable during the replicative phase and appeared only after confluency was attained. In the HM7 line, tyrosinase activity was present at the time of cell planting and declined in inverse proportion to replicative activity. Such dilution of tyrosinase activity with cell replication has been observed in chick melanocyte cultures and has been interpreted as the result of the redistribution of a fixed amount of preformed tyrosinase among an increasing number of newly produced melanocytes (25). This seems to be a reasonable explanation for the results we have described here for the HM7 line. With the attainment of confluency, additional tyrosinase activity is demonstrable and continues to increase each day in a relatively fixed number of cells. This suggests *de novo* enzyme synthesis, but we have not directly examined this point. In contrast, in the B16C3 mouse melanoma cell line, tyrosinase activity appears only after confluency and rapidly diminishes to 0 within a few days. The loss of activity in the B16C3 may be due to a saturation of the melanosomes with accumulated melanin (22). Since the HM7 cells produce very little melanin, such saturation of the melanosomes probably does not occur within the duration of the experiment.

Implantation of HM7 cells into athymic nude mice produced small plaque-like nodules of persistent tumor cells that did not grow progressively. Although a number of human neoplastic cell lines have formed tumors in athymic mice (7), failure to do so cannot be interpreted as evidence that the lines are not neoplastic since other human tumor cell lines implanted in nude mice have also failed to produce progressive growths (23). This could be attributable to inadequate nutritional conditions for these human tumor cells in the athymic mice. Microscopic examination of the persistent nodules in our experiment demonstrated cells with typical human melanoma morphology that were actively invading between the muscle bundles of the underlying panniculus carnosus.

It is not probable that the HM7 line has been contaminated by admixture with murine melanoma cells. The kinetics of growth *in vitro* is very different. The doubling time of the B16C3 line is 19 to 29 hr, while that of the HM7 line is 40 to 72 hr. The kinetics of tyrosinase activation also is remarkably distinctive, the mean cell volumes are at least 2-fold different, and the morphology of both lines is unique. Melanin produced by the B16C3 cells is coal-black in color, while that produced by the HM7 is amber-brown. The chromosomes of murine cells generally are predominantly acrocentric, while those of the HM7 line are metacentric.

The polysaccharides produced by the HM7 human melanoma line differ in several ways from those made by human embryonic iris melanocytes. The percentage of HA made by the HM7 cells is significantly reduced, while the proportion of HS and C4S is significantly increased, and the HM7 cells contain no detectable dermatan sulfate while the human iris melanocytes produce significant quantities. It is not possible to attribute these differences to the neoplastic state alone, as the comparisons are not just between neoplastic *versus* normal melanocytes. The cells studied also differ as adult *versus* embryonic, integumentary *versus* ocular, and established cell line *versus* primary explant. There are no control cell culture lines of normal adult melanocytes. The use of primary explants of human embryonic iris, while inadequate in the respects detailed above, provides the only available control for these studies.

The HS secreted into the medium by the melanoma is apparently incorporated in molecules of higher molecular weight than those previously observed in mouse melanoma (19). The significance of this is not clear and may be a

Table 5
*Properties of the 1.2 M NaCl fractions*

| | M.W. | $GluNH_2$ | $GalNH_2$ | Chondroitinase ACII | Heparitinase |
|---|---|---|---|---|---|
| HM7 media | 35,000+ | 50 | 50 | 58 | 52 |
| H.I.M. media | 35,000–14,000 | 10 | 90 | 90 | |

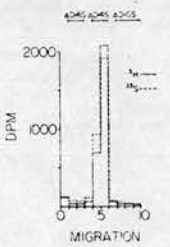


Chart 7. Results of chondroitinase AC digestion and paper chromatography of the 2 M NaCl eluate of the human melanoma media fraction and of chondroitinase ABC digestion of the human iris media fraction 2 M NaCl eluate. *ΔDi6S*, Δ-disaccharide-6-sulfate; *ΔDi4S*, Δ-disaccharide-4-sulfate; *ΔDi0S*, Δ-disaccharide-0-sulfate.

Table 6
*Properties of the 2 M NaCl fractions*

| | | | | Chondroitinase | |
|---|---|---|---|---|---|
| | M.W. | $GluNH_2$ | $GalNH_2$ | ACII | ABC |
| HM7 media | 35,000+ | | 100 | 100 | |
| H.I.M. media | 35,000+ | | 100 | 62 | 100 |

Table 7
*Saccharide products of melanocytes*

| | HA | C4S | Heparan-$SO_4$ |
|---|---|---|---|
| Melanoma | 21.8 | 47.2 | 31.1 |
| Normal iris[a] | 79.3 | 19.0[b] | 1.0[c] |

[a] About 10% of the [$^3$H]$GlcNH_2$ counts in the 0.8 M media and cell-associated fraction could not be completely characterized due to the small number present.

[b] Includes dermatan-$SO_4$, 5.6%.

[c] Figure given assumes that all of the [$^3$H]$GlucNH_2$ counts in the 1.2 N media fraction are in HS.

species difference or may reflect the existence of hybrid polysaccharide structures. The overall results are similar to those previously reported on the B16 mouse melanoma 1921), indicating a reduction in the amount of HA and an increase in the percentage of high-molecular-weight C4S and HS. The major differences between the lines are the lack of dermatan-$SO_4$ production by the mouse iris melanocytes, the presence of a lower molecular weight HS in the mouse melanoma, and the complete absence of HA production by the mouse melanoma. Similar effects indicating the reduction of HA synthesis by transformation have been shown for SV40-transformed mouse or human fibroblasts (8, 10), but the opposite effect has been shown to occur for hamster cells transformed by herpes simplex virus (20).

The biological function of the complex saccharide molecules described and their intracellular location are not known. Lectin-binding sites increase in number after hyaluronidase treatment of Rous sarcoma virus-transformed CEF cells (5). Treatment of HeLa cells with chondroitinase ABC produced a marked reduction in electrophoretic mobility, indicating that these molecules are associated with the cell surface (24). However, it is difficult to determine if these molecules are anchored into the plasma membrane and/or are cell surface associated through ionic or protein core interactions. In addition, the presence of polysaccharides in the nucleus has been reported (3).

The reduction of HA synthesis and the increase in HS and $CSO_4$ synthesis may represent compensatory responses. These molecules (HS, $CSO_4$) are markedly different from HA in physical parameters, and one might reasonably expect some changes in surface properties to occur, especially if the amount associated with the cell surface changes significantly. The distribution of these components on the surface as well as their variation during the cell cycle are also of interest.

The recent report of an alkali-stable common melanoma antigen being excreted by the majority of human melanoma patients (6) raises the possibility that the protein component of 1 of the saccharide proteoglycans functions as an antigen; alternative sources may be glycoproteins shed from the cell surface.

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# CHARACTERISTICS OF A MUCIN-TYPE SIALOGLYCOPEPTIDE PRODUCED BY B16 MOUSE MELANOMA CELLS

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Received March 8,1976

SUMMARY: The glycopeptides produced by B16 mouse melanoma cells grown in the presence of [$^3$H]glucosamine were isolated and fractionated into two classes (I and II) with cetyl pyridinium chloride. The class I glycopeptides were of higher molecular weight and of higher negative charge (sialic acid content) than those in class II. Class I glycopeptides contained N-acetyl neuraminic acid, galactose and N-acetylgalactosamine and on treatment with alkaline-borohydride were degraded to apparently tri- and tetrasaccharides. The presence of this mucin-type glycoprotein on the cell surface was detected by mild trypsinization of intact cells.

Examination of proteins and glycoproteins of control and transformed cells by gel filtration or by gel electrophoresis has shown numerous differences of a quantitative nature. These include a sialoglycopeptide derived from membranes (1,2) and a high molecular weight surface protein that disappears on transformation (2-4).

While studying the polysaccharides produced by B16 mouse melanoma cells it was discovered that cetyl pyridinium chloride (CPC)[1] was capable of precipitating a sialoglycopeptide from the pronase digested cells and from spent media in which these cells were grown. Markedly reduced amounts of this sialoglycoprotein were produced by a control population of mouse iris melanocytes (5).

This study describes the partial characterization of this sialoglycopeptide. Its properties are compared with those of the glycopeptides which are not precipitated by CPC and remain in the supernatant.

[1] Abbreviations used: CPC, cetyl pyridinium chloride; TPCK-trypsin, L-(1-tosylamido-2-phenyl)-ethyl chloromethylketone treated trypsin; GalNAc, N-acetyl galactosamine.

## MATERIALS AND METHODS

B16 mouse melanoma cells were grown and the complex saccharides labeled with [$^3$H]glucosamine and $Na_2{}^{35}SO_4$ as described previously (5,6).

The glycopeptides were isolated from labeled cells as well as the spent media by exhaustive digestion with pronase and fractionation of the solubilized components with CPC (6). For isolation of cell surface complex saccharides, cells were cultured for 48 hours prior to harvest in medium containing 1/3 the usual amount of glucose and 0.5 μCi $ml^{-1}$ of D-[$^{14}$C]glucosamine (New England Nuclear, 51.5 mCi $mmole^{-1}$). The cells were subjected to short-term successive incubations with TPCK-trypsin (Worthington Biochemical Corp.) according to Codington *et al* (7) and the resulting trypsinates digested with pronase to yield cell surface glycopeptides.

Hexosamine determinations on isotopically labeled glycopeptides were carried out on acid hydrolysates (4 N HCl, 8 hr, 100°C) on a Beckman 120C amino acid analyzer employing a stream-splitting attachment. Unlabeled and [$^{14}$C]-labeled glycine, glucosamine and galactosamine were used as markers. To identify hexosaminitols, the citrate-borate buffer system as described by Bella and Kim (8) was used. Labeled sialic acid in glycopeptides was determined either by acid hydrolysis (0.1 N $H_2SO_4$, 80°C, 1 hr) or neuraminidase treatment followed by separation of sialic acid from asialoglycopeptide on a BioGel P2 200-400 mesh column (0.9 x 70 cm) by elution with 0.1 M pyridine acetate, pH 5.0. Radioactivity in column fractions was determined as described previously (6). To locate radioactivity on paper chromatograms, 1 cm paper strips were extracted with 1 ml water in counting vials prior to addition of scintillation liquid and counting. Paper chromatography was done on Whatman No. 1 paper using the following solvents: A, N-butyl acetate:glacial acetic acid:water (3:2:1); B, N-butyl alcohol:pyridine:water (6:4:3); C, ethylacetate:pyridine:water (9:3:2). Marker sugars were detected by a silver nitrate stain.

*Vibrio cholerae* neuraminidase (Calbiochem) digestion was done in 0.1 M Na acetate buffer, pH 5.6 containing 0.001 M $CaCl_2$ with 188 milliunits of enzyme in a total digest volume of 200 μl; incubation was performed at 37°C. Influenza virus neuraminidase (Calbiochem) digestion was carried out in 0.05 M Tris-maleate buffer, pH 6.5 containing 0.001 M $CaCl_2$ with 174 milliunits of the enzyme in a volume of 200 μl. Incubation with β-galactosidase from Jack bean (9) was performed in 0.05 M citrate-buffer, pH 4.0 in a volume of 50 μl using 0.1 units enzyme for 24 hr at 37°C. Digestions with β-N-acetyl hexosaminidase and with α-N-acetyl galactosaminidase both from *Charonia lampus* (Miles Laboratories) were also done under the above conditions using 0.1 unit or 10 milliunit of the enzymes respectively. The released labeled sugars were estimated by chromatography of the digest on BioGel P2 columns as described above.

## RESULTS AND DISCUSSION

The labeled glycopeptides synthesized by the mouse melanoma and present in the pronase digest of the cells or media were fractionated into two classes on the basis of their interaction with CPC. The Class I glycopeptides were selectively eluted from the CPC precipitate with 0.2 M NaCl, whereas the Class II glycopeptides which remain in the CPC supernate were isolated after precipitation of CPC with potassium thiocyanate and extensive dialysis. The Class I glycopeptides contained 4-5% of the incorporated tritium label and those in Class II about 90%; the balance of the tritium

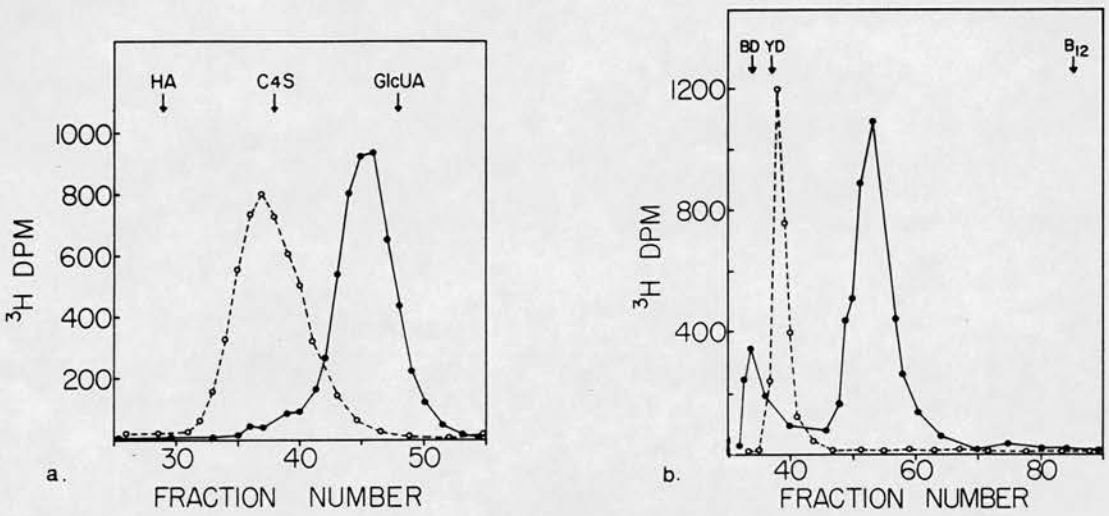


Fig. 1: Chromatography of mouse melanoma glycopeptides (a) on CPG 10-240 glass bead column (0.9 x 90 cm). Elution was with 0.5 M $CaCl_2$ at 30 ml per hr. The peak elution positions of marker saccharides (HA; vitreous humor hyaluronic acid; C4S porcine rib cartilage chondroitin-4-sulfate and GlCUA, glucuronic acid) determined by the oricinol reaction are indicated by arrows. (b) On Sephadex G-50 column (0.9 x 140 cm) eluted with 0.1 M pyridine acetate pH 5.0. The peak elution positions of markers BD, blue dextran 2000; YD, yellow dextran and Bl2, vitamin $B_{12}$ are indicated by arrows. One (1) ml fractions were collected and aliquots analyzed for radioactivity. Class I (---O---O---) and Class II (—●—●—) glycopeptides were run in separate experiments and the elution patterns superimposed.

label and virtually all of the non-dialyzable $^{35}S$ label were in mucopolysaccharides.

The Class I glycopeptides were of higher molecular weight (Fig. 1) and higher negative charge (Fig. 2) than those in Class II. The former appeared relatively homogenous on the basis of size and charge whereas the latter was obviously a complex mixture. The tritium label in these glycopeptides was distributed between sialic acid and hexosamines. Class I glycopeptides had about 38% of their label in sialic acid, the balance being in galactosamine. The label in Class II glycopeptides was distributed between sialic acid (25%), glucosamine (66%) and galactosamine (9%). The sialic acid of glycopeptide I was identified as [$^3$H]-N-acetyl neuraminic acid by co-chromatography with standard on a BioGel P2 column, by paper chromatography in

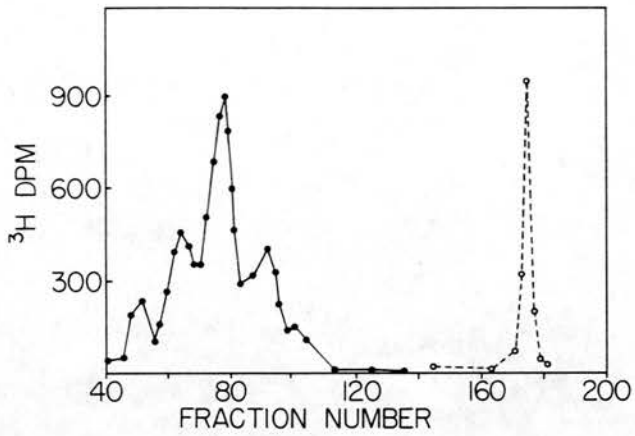


Fig. 2: Chromatography of Class I and II glycopeptides from mouse melanoma cells on DEAE cellulose column (0.9 x 70 cm). Elution was with a linear gradient of 0.01 M to 0.5 M pyridine acetate buffer, pH 5.1 (fractions 1-140), followed by 1 M pyridine acetate of same pH. Rate of flow was 30 ml per hr. Fractions of 2.5 ml were collected and aliquots analyzed for radioactivity. Class I (---O---O---) and Class II (—●—●—) were analyzed on separate runs and the results are presented by superimposing the elution patterns.

solvent A and by paper electrophoresis (3 MM paper, pyridine acetate buffer, pH 3.5, 10V/cm, 3 hr). Further evidence that the hexosamine of glycopeptide I is galactosamine was obtained by ninhydrin oxidation (10) followed by identification of [$^3$H]-lyxose as the only labeled component by paper chromatography (solvent C). [$^3$H]-galactose was the only neutral sugar detected by paper chromatography of a total acid hydrolysate of Class I glycopeptides isolated from cells grown in the presence of [$^3$H]glucose (V. P. Bhavanandan, J. Umemoto, J. Banks and E. A. Davidson, unpublished results).

*Vibrio cholerae* neuraminidase digestion of Class I glycopeptides released virtually 100% of the labeled sialic acid in 24 hr, indicating that all the sialic acid was present as terminal residues. However, influenza virus neuraminidase released only about 50% of the sialic acid in 24 hr, suggesting that sialic acid may be involved in more than one type of linkage. It has been reported that whereas *Vibrio cholerae* neuraminidase acts equally on both 2→3 and 2→6 linked sialic acid, influenza virus

enzyme acts preferentially on 2→3 and only weakly on 2→6 linkage (11).

Incubation of the asialoglycopeptide (prepared by *Vibrio cholerae* neuraminidase treatment) with *Charonia lampus* β-hexosaminidase, *Charonia lampus* α-N-acetyl galactosaminidase or Jack bean β-galactosidase followed by β-hexosaminidase failed to release any labeled sugar. However, when the asialoglycopeptide was treated with Jack bean β-galactosidase followed by α-N-acetyl galactosaminidase, a component with 27% of the label was released and was identified as N-acetyl galactosamine by paper chromatography in solvents A and B. This suggests the presence of the sequence $Gal \xrightarrow{\beta} [^3H]GalNAc \xrightarrow{\alpha}$ in the asialoglycopeptide.

In order to examine the nature of the linkage between carbohydrate and peptide, the Class I glycopeptides were treated under nitrogen with 0.3 M $NaBH_4$ - 0.1 N NaOH for 90 hr at 37°C in sealed tubes in the dark. The excess borohydride was destroyed by careful addition of 1 N acetic acid and the neutralized solution chromatographed on controlled pore glass bead column. The radioactive material now migrated in the low molecular weight region with the peak elution position at fraction 48 (Fig. 1a). On BioGel P2 and P4 columns, about 90% of the radioactive material was included whereas the untreated sample was entirely excluded. Paper chromatography of the alkali degraded product gave two spots, the major one having $R_{Lactose}$ 0.34 and 0.09 and the minor one 0.79 and 0.56 in solvent systems A and B respectively. On hydrolysis of the β-elimination products, only [$^3$H]galactosaminitol was detectable with virtually no [$^3$H]galactosamine indicating that only one galactosamine per prosthetic group was present and that this was involved in an alkali-labile O-glycosidic linkage apparently to serine and/or threonine.

The class I glycopeptide is rich in sialic acid and galactosamine, free of glucosamine and of higher molecular weight than the other glycopeptides produced by melanoma cells. It is distinct from the peak A glycopeptides described by Warren and co-workers (1,12) since these glycopeptides have been shown to have the serum-type N-glycosidic linkage (13). Even though an

accurate estimation of the size of the prosthetic group has not been made, its mobility on BioGel columns and paper chromatograms indicate a mixture of tetra- and trisaccharides. The enzymatic digestion data are consistent with the sequence Gal$\xrightarrow{\beta}$GalNAc$\xrightarrow{\alpha}$Ser/Thr. To this core, one or two sialic acid residues are probably attached, forming tri- or tetrasaccharide units. In the case of the later, the two sialic acid residues are most likely attached by two types of linkages (e.g. 2→3 and 2→6). The glycopeptide appears to be of molecular weight 10-15,000 on the basis of its behavior on controlled pore glass and Sephadex G-50 columns in comparison with standard anionic saccharides. Thus it is conceivable that several oligosaccharides are present in a cluster attached to the peptide core resulting in a molecule resistant to further proteolytic digestion.

When the mouse melanoma $^{3}$H-glycopeptides isolated from the media were co-chromatographed together with the [$^{14}$C]-glucosamine labeled components isolated from the surface of intact mouse melanoma cells, strikingly similar profiles were obtained both on molecular sieve (CPG; Sephadex G-50) as well as ion exchange (DEAE cellulose) columns (not illustrated). This suggests that the Class I and II glycopeptides are located at least partly at the cell surface. These components may be anchored in the membrane or they may reside only temporarily in the glycocalyx (cell coat) in the process of being shed into the medium.

The production of this glycoprotein by melanoma cells but not by control melanocytes (5) is of extreme interest. Mucin-type glycoproteins have been isolated from the membranes of mammalian erythrocytes (14,15) and of cancerous cells grown in ascites form (16-18), whereas solid tissue controls (19) did not make comparable glycopeptides. The production of this class of glycoproteins by substratum grown mouse melanoma as well as human melanoma cells (20; V. P. Bhavanandan, J. Banks and E. A. Davidson, unpublished results) seems to indicate that this may be a more general characteristic of cancer cells.

ACKNOWLEDGEMENT: We would like to thank Mses. Debora Breindel, Ellen Goldberg and Mr. Mord Moore for their assistance in this work and Dr. Y-T. Li for a gift of Jack bean β-galactosidase. These studies were supported in part by Grant CA15483 from the U. S. Public Health Service.

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# Characterization of an Endo-α-N-Acetyl Galactosaminidase from *Diplococcus Pneumoniae*

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Received April 1,1976

SUMMARY: Evidence is presented for the presence in filtrates of *Diplococcus pneumoniae* of an endo-glycosidase capable of acting on the O-glycosidic linkage between N-acetyl galactosamine and serine or threonine residues. The glycosidase was partially purified by chromatography on Affi-Gel 202. The resulting preparation acted on glycopeptides from mouse melanoma, fetuin and pig submaxillary mucin to release a disaccharide characterized as galactosyl-N-acetyl galactosamine. The enzyme had no action on phenyl α-N-acetyl-D-galactosaminide, asialo ovine submaxillary mucin or monosialoganglioside. A similar activity was detected in a commercial preparation of *Clostridium perfringens* neuraminidase.

Endo-glycosidases would be extremely useful tools for structural studies on glycoproteins and for investigating the nature and function of cell surface complex carbohydrates. Endo-β-N-acetyl glucosaminidase activity, first demonstrated by Muramutsu (1) in *Diplococcus pneumoniae*, has since been isolated from various microorganisms (2-4). This enzyme hydrolyzes the di-N-acetyl-chitobiose linkages in glycoproteins having a glucosaminyl asparagine bond; the exact substrate specificity depends on the source of the enzyme (2-4). The presence of α-N-acetyl galactosaminyl oligosaccharase in a crude enzyme preparation from *Clostridium perfringens* has been reported (5). The present report describes some of the properties of such a glycosidase obtained from *D. pneumoniae*.

Chromatographic fractionation of glycosidases from *D. pneumoniae* gave a preparation of neuraminidase with very high specific activity. However, on digesting a labelled sialoglycopeptide from mouse melanoma cells with this enzyme fraction, it was discovered that in addition to sialic acid, an oligosaccharide was released. Structural analysis of this oligosaccharide

resulted in the identification of an endo-glycosidase which in contrast to the enzyme described by Muramutsu acts on the O-glycosidic linkage between α-N-acetyl galactosamine and serine or threonine in glycoproteins.

## MATERIALS AND METHODS

The crude preparation of enzymes isolated from the culture filtrates of *Diplococcus pneumoniae* type 1 and having neuraminidase, β-N-acetyl hexosaminidase, β-galactosidase and endo-β-N-acetyl glucosaminidase activities but no proteases was generously provided by Dr. Gilbert Ashwell.

Mouse melanoma sialoglycopeptides (class I) were isolated from [$^3$H]-glucosamine labelled cells as described (6,7). The tritium label in this preparation was distributed between sialic acid (40%) and galactosamine (60%). Pig submaxillary mucin (PSM)[1] isolated from pooled glands was a gift from Dr. M. de Salegui. Glycopeptides from this glycoprotein were isolated as described (8), except that the pronase digest was ultrafiltered through a $UM_2$ membrane (Amicon) before chromatography on Sephadex G-50. The anthrone positive material consisting of the mixed glycopeptides was used in these studies. Fetuin was purchased from Gibco and the O-glycosidically linked glycopeptides from it were prepared as described by Spiro and Bhoyroo (9), with the following modification: the pronase digest was ultrafiltered using a $UM_{0.5}$ membrane (Amicon) and directly fractionated on a Sephadex G-25 (2.6 x 128 cm) column. The glycopeptides corresponding to Fraction C of Spiro and Bhoyroo were used in these studies; 36 mg was obtained from 1 g fetuin. Ovine submaxillary mucin and asialo ovine submaxillary glycopeptides were isolated as described previously (10). Neuramin-(2→3)-lactose was isolated from skimmed bovine colostrum as described previously (11).

Activities of β-galactosidase and β-N-acetyl hexosaminidase were measured using O-nitrophenyl-β-D-galactopyranoside and p-nitrophenyl-N-acetyl-β-D-glucosaminide (both from Calbiochem) respectively, as described (12). α-N-acetyl-D-galactosaminidase was assayed using either phenyl-N-acetyl-α-D-galactosaminide (Nakarai Chemicals, Japan) or asialo ovine submaxillary glycopeptides as substrate. The incubation mixtures were assayed for N-acetyl galactosamine by the Morgan-Elson reaction (13) and in the case of the former substrate also by Folin-Ciocalteu reaction for phenol. The incubation mixture for the neuraminidase assay consisted of 25 µl of 0.1 M phosphate-citrate buffer, pH 6.5 (12), 50 µl of a 5 mM solution of Neuramin-(2→3)-lactose and enzyme in a final volume of 200 µl. One unit of neuraminidase activity is defined as the amount of enzyme that causes the release of 1 µmole of NANA per 30 min. After incubation at 37°C for 30 min, the released sialic acid was measured by a modification of the thiobarbituric acid method (14). Incubation with the endo-α-N-acetyl galactosaminidase was done under the same conditions as for neuraminidase using the appropriate substrates. After the incubation, aliquots of the mixture were assayed for Morgan-Elson positive material. The product was further identified by gel filtration on BioGel P2 <400 mesh columns (0.9 x 64 cm) and by paper chromatography.

Paper chromatography was done on Whatman No. 1 paper using the following solvents: A, N-butyl acetate:glacial acetic acid:water (3:2:1); B,N-butyl alcohol:pyridine:water (6:4:3). Radioactivity in column fractions and on paper chromatograms was determined as described previously (6,7).

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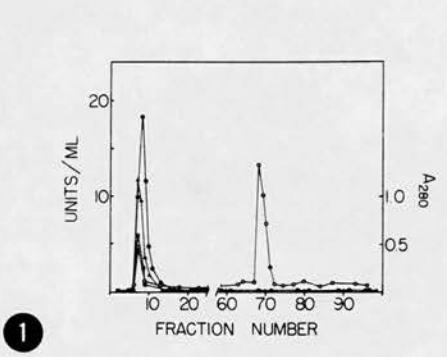
[1] Abbreviations used: PSM, pig submaxillary mucin; GalNAc, N-acetyl galactosamine, NANA, N-acetyl neuraminic acid.

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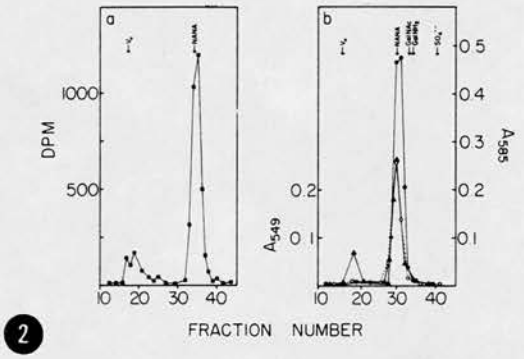


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Figure 1. Chromatography of *Diplococcus pneumoniae* crude enzyme preparation on Affi-Gel 202 at 4°C. The column (1.2 x 26 cm) was eluted with 0.02 M Tris-HCl buffer, pH 7.0 and fractions of 1.5 ml were collected. The eluant was changed to 0.5 M NaCl in the same buffer at fraction 62. The fractions were analyzed for ultraviolet absorbance at 280 (—■—■—), β-N-acetyl-D-hexosaminidase (—●—●—), β-galactosidase (—▲—▲—) and neuraminidase (—○—○—) as described under "Methods". The scale for neuraminidase activity is 1/20 of that of the other enzymes.

Figure 2. Fractionation of enzyme fraction II digests of a) [$^3$H]-asialoglycopeptides from B16 mouse melanoma; b) fetuin glycopeptide fraction C on BioGel P2 <400 mesh columns. The columns were eluted with 0.1 N pyridine acetate at the rate of 30 ml per hr and fractions of 1 ml were collected. Aliquots were analyzed for tritium radioactivity (—■—■—), Morgan-Elson reacting material (—●—●—), free sialic acid (—○—○—) and total sialic acid after acid hydrolysis (—▲—▲—). Void volume (Vo) and peak elution positions of standard sugars and inorganic sulfate are indicated by arrows.

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Isolation of the endo-enzyme: The crude enzyme was placed on a column of Affi-Gel 202 (BioRad) and the column eluted with 0.02 M Tris-HCl buffer, pH 7.0 followed by 0.5 M NaCl in the same buffer in the cold room, 4°C. The result of the fractionation is illustrated in Figure 1.

## RESULTS AND DISCUSSION

The designated fractions in Figure 1 were combined and concentrated by ultrafiltration. The concentrated enzyme fractions I and II were tested for glycosidases and proteases. Fraction I had β-galactosidase, β-N-acetyl hexosaminidase and neuraminidase activities. The only detectable exo-glycosidase activity in fraction II was neuraminidase; the protein in this fraction was very low and there was no protease activity when tested against Azocoll (Calbiochem).

Treatment of mouse melanoma class I [$^3$H]-sialoglycopeptide with enzyme fraction II followed by chromatography on a BioGel P2 column released

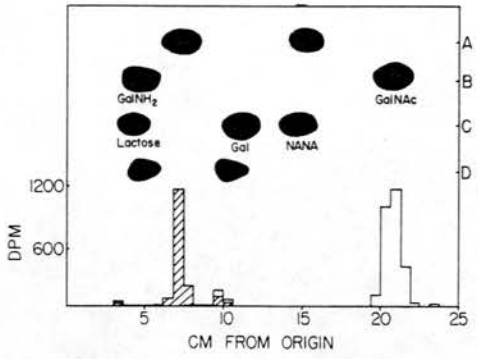


Figure 3. Paper chromatography of low molecular weight material isolated by BioGel P2 column chromatography of enzyme fraction II digests. The position of the labelled oligosaccharide from mouse melanoma asialoglycopeptides (fractions 33-37, Figure 2a) is indicated by the shaded area. After treatment with Jack bean β-galactosidase the labelled material (unshaded area) has the same mobility as GalNAc. The low molecular weight material from fetuin glycopeptide (fractions 29-33, Figure 2b) before (lane A) and after (lane D) acid hydrolysis (3N HCl, 100°C, 6 hr). Lanes B and C are standards.

about 90% of the radioactivity as low molecular weight material. This was unexpected since only about 40% of the radioactivity was in sialic acid was determined by mild acid hydrolysis (0.1 N $H_2SO_4$, 80°, 1 hr). Treatment of [$^3$H]-asialoglycopeptide, prepared by *Vibrio cholerae* neuraminidase digestion, with fraction II for 24 hrs, released 73% of the radioactivity (Figure 2). Paper chromatography in solvent A of the low molecular weight material released from the sialoglycopeptide showed two spots, one with the mobility of N-acetyl neuraminic acid and the other with a lower $R_F$ was apparently an oligosaccharide (Figure 3). The material released from the asialoglycopeptide showed only the oligosaccharide on paper chromatography. On rechromatography of the product from asialoglycopeptide, after treatment with Jack bean β-galactosidase, a labelled spot with the mobility of N-acetyl galactosamine was detected (Figure 3). These results indicated that an enzyme in fraction II was releasing an oligosaccharide with the structure $(Gal)_x 1 \xrightarrow{\beta} GalNAc$. The class I mouse melanoma glycopeptide has a mucin type structure in which the prosthetic group is linked to serine

and/or threonine through α-N-acetyl galactosaminyl residues (7); therefore, it appeared that an endo-glycosidase in fraction II was cleaving this linkage. In order to confirm this finding the action of this enzyme on O-glycosidically linked glycopeptides from fetuin and PSM was tested.

Digestion of fetuin glycopeptide fraction C with this enzyme resulted in the production of a Morgan-Elson positive material. In order to identify this product the incubation mixture was chromatographed on a Bio-Gel P2 column (Figure 2). Two peaks were obtained; fractions in the second peak reacted with Morgan-Elson and thiobarbituric acid reagents, whereas fractions in the first peak reacted with neither but reacted with the latter reagent after acid hydrolysis (0.1 N $H_2SO_4$, 80°C, 1 hr). Chromatography of a control digest, without enzyme, on the same column gave only the first peak. The material eluting in the second peak was isolated and on paper chromatography (solvents A and B) showed two silver nitrate staining components with the same mobilities as N-acetyl neuraminic acid and the oligosaccharide from mouse melanoma glycopeptide (Figure 3). The oligosaccharide was separated from sialic acid by chromatography on an AG1 (formate) resin column. Acid hydrolysis (3 N HCl, 100°, 6 hr) of the oligosaccharide gave galactose and galactosamine, identified by paper chromatography (Figure 3). Quantitative analysis indicated the presence of equimolar amounts of these sugars in the hydrolysate; galactose was determined by anthrone reaction (15) and galactosamine by the Elson-Morgan reaction (16). Reduction of the oligosaccharide with sodium borohydride followed by acid hydrolysis gave galactose and galactosaminitol as identified by chromatography on borate treated paper in solvent A. These results prove that an enzyme activity in fraction II is capable of releasing the disaccharide galactosyl-(1→3)N-acetyl galactosamine from fetuin glycopeptide fraction C.

Submaxillary glycopeptides on digestion with the enzyme fraction II gave a Morgan-Elson positive product which was isolated by BioGel P2

column chromatography and identified as the disaccharide, galactosyl-N-acetyl-galactosamine, as described above. The same product was obtained when PSM glycopeptides from which fucose and sialic acid had been removed by mild acid hydrolysis (1 M HCOOH, 100°C, 1 hr) and the galactosamine re-N-acetylated (17) was used as substrate. The PSM preparation was from pooled glands and thus probably has both blood group $A^+$ and $A^-$ active components. The isolation of only a disaccharide and not higher oligosaccharides from digests of the PSM glycopeptides indicates that the enzyme might require an unsubstituted galactose for activity. It will be interesting to determine whether this enzyme is capable of releasing sialo-oligosaccharides, such as sialyl-N-acetyl galactosamine from ovine submaxillary glycopeptides. This can be determined once the enzyme is purified free of neuraminidase.

The enzyme also released the same disaccharide from intact PSM and PSM from which fucose and sialic acid had been removed and the galactosamine re-N-acetylated. It is thus capable of acting on intact glycoproteins, a property which will be very valuable in structural studies on glycoproteins. The enzyme fraction II did not have any action on the following: phenyl-α-N-acetyl galactosaminide, asialo ovine submaxillary glycoprotein and its glycopeptides, monosialoganglioside ($GM_1$) and asialo $GM_1$ prepared by acid treatment (1 M HCOOH, 100°, 1 hr). It is thus apparent that it has no exo-N-acetyl galactosaminidase activity and the endo activity is specific for the α-linkage, since in $GM_1$ (Gal→GalNAc) is linked β to galactose.

Using class I [$^3$H]-sialoglycopeptides from mouse melanoma we were able to detect the same endo-glycosidase activity in a commercial preparation (Worthington) of *Clostridium perfringens* neuraminidase.

The evidence reported in this communication firmly establishes the presence of an endo-glycosidase, identified as endo-α-N-acetyl galactosaminidase, in a fraction obtained from *D. pneumoniae* culture filtrates. The only other detectable enzyme activity in this fraction is neuraminidase.

The binding of neuraminidase and the endo-enzyme to Affi-Gel 202 followed by their elution with NaCl is of interest. Affi-Gel 202 is an agarose gel bead to which the spacer arm $-\overset{\overset{NH}{|}}{C}O\,NH(CH_2)_3NH(CH_2)_3NHCO(CH_2)_2COOH$ is attached. This support is used for immobilizing ligands for affinity chromatography. *D. pneumoniae* neuraminidase also bound to Affi-Gel 202 to which p-aminobenzyl-1-thio-2-acetamido-2-deoxy-β-D-glucopyranoside was coupled (J. Umemoto, V. P. Bhavanandan and E. A. Davidson, unpublished results). This indicates that the neuraminidase is bound to the Affi-Gel 202 by non-specific (hydrophobic) interaction with the spacer arm, and not to the carboxyl group.

In preliminary experiments the crude enzyme preparation was chromatographed on a Sepharosyl-glycyltyrosyl-[N-(p-aminophenyl)]oxamic acid column (0.8 x 2.8 cm) as described by Den *et al.* (18). The neuraminidase activity was separated from β-galactosidase and β-N-acetyl hexosaminidase but the endo-enzyme was detected in the neuraminidase containing fraction. It was noticed that on storage, the activity of the neuraminidase in the fraction obtained from Affi-Gel 202 column decreased more rapidly than that of the endo-enzyme. Partial separation of the neuraminidase and endoenzyme activities was also obtained when the crude preparation was chromatographed on Affi-Gel 202 using a NaCl gradient for elution. These results indicate that neuraminidase and the endoenzyme activities are two separate enzymes. The possibility of using these properties to separate the two enzyme activities is being explored. Purification of the endoenzyme as well as the properties of this enzyme, such as pH optimum and the exact substrate specificity, will be reported in a subsequent publication.

ACKNOWLEDGEMENTS: We are most grateful to Dr. Gilbert Ashwell for providing generous quantities of the crude enzyme preparation used in these studies. We also thank Dr. M. de Salegui for pig submaxillary mucin, Dr. Y-T. Li for Jack bean β-galactosidase, Dr. H. Den for the affinity column of N-(p-aminophenyl)oxamic acid, Dr. C.-L. Schengrund for monosialoganglioside and Mr. Jeffery Kemper for technical assistance. These studies were supported in part by grant CA 15483 from the U.S. Public Health Service.

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Separatum EXPERIENTIA *32*, 1501 (1976)
Birkhäuser Verlag, Basel (Schweiz)

# Interaction Between the Soluble and Particulate Neuraminidases of Chick Liver[1]

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*Summary.* The sum of the neuraminidase activities found in the isolated soluble and particulate fractions of chick liver was considerably higher than that observed in the cytoplasmic extract from which these fractions were obtained. Addition of increasing amounts of particulate neuraminidase to a constant amount of the soluble preparation resulted in a progressive loss of enzyme activity.

Chick liver, as the liver of other vertebrates, contains both a soluble and a particle-bound neuraminidase[4]. Unlike the situation observed with rat liver[5], the chick liver neuraminidases have rather close pH optima: 3.8 for the particulate and 4.4 for the soluble enzyme[4].

While investigating the intracellular distribution of this enzyme, it was observed that the sum of the neuraminidase activities found in the isolated soluble and particulate fractions was more than 50% higher than that of the cytoplasmic extract from which these fractions were derived. In order to further investigate this observation, chick liver homogenates (1:4 w/v) were prepared in 0.154 *M* KCl and nuclei and debris were removed by low speed centrifugation (10 min at 800 *g*). Neuraminidase activity was then measured in this cytoplasmic extract as well as in the soluble fraction and in the sedimentable fraction (resuspended in 0.154 KCl) obtained from it by centrifuging for 1 h at 105,000 *g*. All neuraminidase assays were conducted at pH 4.0; at this pH, about 95% of the maximum activity for the soluble and particulate fractions can be detected[4]. The *N*-acetylneuraminic acid (NANA) released by the action of the enzyme was measured by the thiobarbituric acid method[6].

In a series of determinations conducted using as substrates various sialyltrisaccharides isolated from rat mammary gland[7] and from cow colostrum[8], the neuraminidase activity in the cytoplasmic extract was consistently lower than the sum of the values obtained for the soluble and particulate fractions (see table).

Addition of increasing amounts of the particulate neuraminidase to a constant amount of the soluble preparation resulted in a progressive loss of neuraminidase activity (see figure). When the two enzymes are present in the mixture in the same proportion found in the original tissue, the activity of the mixture is usually 50–70% of that given by the soluble neuraminidase by itself. Heat inactivated (15 min at 100 °C) particulate neuraminidase was less effective but, nevertheless, clearly inhibitory.

Several possible mechanisms that could account for this inhibition were investigated and the results are summarized below.

Addition of ashes from 1 g of whole fresh liver to the soluble fraction from 1 g of liver caused much less inhibition (less than 20%); at least one half of the inhibition caused by the ashes is probably due to the inhibitory effect of chlorides on the soluble neuraminidase[4].

[1] This investigation was supported in part by U. S. Public Health Service Grant NS09176 from the National Institutes of Health.
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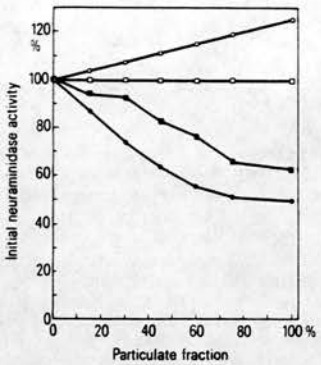
Neuraminidase activity in the soluble and particulate fractions and in the unfractionated cytoplasmic extract prepared from the livers of 8-day-old white leghorn chicks

| Substrate: type of linkage and source | Enzyme preparation | Neuraminidase activity | | | |
|---|---|---|---|---|---|
| | | nmoles NANA/h · g of liver | | | |
| Neuramin-lactose | Cytoplasmic extract | | 247 | | 205 ± 33 |
| α2→3′ | Soluble fraction | 318 | (396) | 292 ± 61 | (395 ± 65) |
| Rat mammary gland | Particulate fraction | 78 | | 103 ± 21 | |
| Neuramin-lactose sulfate | Cytoplasmic extract | | 499 | | 613 ± 99 |
| α2→3′ | Soluble fraction | 597 | (752) | 583 ± 57 | (820 ± 129) |
| Rat mammary gland | Particulate fraction | 155 | | 237 ± 92 | |
| Neuramin-lactose | Cytoplasmic extract | | 225 | | |
| α2→3′ | Soluble fraction | 308 | (372) | | |
| Cow colostrum | Particulate fraction | 64 | | | |
| Neuramin-lactose | Cytoplasmic extract | | 112 | | |
| α2→6′ | Soluble fraction | 149 | (184) | | |
| Cow colostrum | Particulate fraction | 35 | | | |

The set of values on the left corresponds to the results obtained from a single representative experiment conducted with the various substrates listed. The mean ± S. D. from seven different experiments with neuramin-lactose, and three experiments with neuramin-lactose sulfate, are shown on the right side of the table. Figures in parentheses represent the sum of the activities of the soluble and particulate fractions.




Inhibition of soluble chick-liver neuraminidase by increasing concentrations of active and heat-inactivated particulate neuraminidase. A constant amount of soluble enzyme was present in all incubation mixtures. Ordinate values represent the activity of the mixtures expressed as percent of the activity given by the soluble fraction by itself. Abcissa values indicate percent of active (●) and heat inactivated (■) particulate fraction added to the mixture; at 100%, soluble and particulate fractions are present in the same proportion found in the original fresh tissue. All assays were conducted using neuramin-lactose (2→3′) as the substrate; the data shown corresponds to the average of two separate experiments. Theoretical values, calculated by adding the activities of the soluble and particulate enzymes assayed separately, are included for mixtures containing active (○) and heat-inactivated ( ) particulate preparations.

No destruction of free NANA (NANA-aldolase activity), or interference with the thiobarbituric acid assay, by the particulate fraction could be detected.

No marked differences were observed in the pH-versus activity curves of the cytoplasmic extract, soluble and particulate enzymes.

No abnormal behavior was observed upon determination of neuraminidase activity with increasing substrate concentrations. The $K_m$ values for neuramin-lactose (2→3′) were of the same order of magnitude: soluble neuraminidase $1.20 \times 10^{-3}$ $M$; particulate neuraminidase $2.72 \times 10^{-3}$ $M$ and cytoplasmic extract $1.97 \times 10^{-3}$ $M$.

Experiments with [$^{14}C$]-U-neuramin-lactose[9] indicated absence of irreversible binding of the substrate by the particulate fraction and ruled out possible transfer (transglycosylation) of NANA (cleaved from the substrate by neuraminidase action) to endogenous or exogenous acceptors.

Since the cytosolic neuraminidase of chick liver exhibits very low activity towards macromolecules[4], our data suggests the possibility that binding of this enzyme to sialyl groups on the surface of the native, and of the heat-inactivated, particulate fraction may account for the loss of activity observed in these experiments. It is tempting to speculate that a similar phenomenon may also play a role in the regulation of neuraminidase activity in vivo.

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Reprinted from

*Biochimica et Biophysica Acta*, 498 (1977) 250—254


BBA 28266

# RELEASE OF *O*-SULFATE GROUPS UNDER MILD ACID HYDROLYSIS CONDITIONS USED FOR ESTIMATION OF *N*-SULFATE CONTENT

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(Received December 20th, 1976)

## Summary

The treatment of chondroitin sulfate isolated from cultured B16 mouse melanoma cells with 0.04 M HCl at 100°C for 90 min released up to 45% of *O*-sulfate residues as free inorganic sulfate. In addition to the release of inorganic sulfate, extensive degradation of this polysaccharide as well as of cartilage chondroitin sulfate, pig rib cartilage proteoglycan, heparin and hyaluronic acid was also evident under these conditions. The above hydrolysis conditions are used for characterizing $^{35}S$-labeled heparan sulfates synthesized by cultured cells and to calculate ratio of *N*- and *O*-sulfates in these molecules. Our results suggest that caution is necessary in interpreting the results of mild acid hydrolysis of glycosaminoglycans.

## Introduction

Sugar sulfates, like most esters, are easily hydrolyzed by both alkalis and acids. The rate of release of sulfate from sulfated sugars during acid hydrolysis depends on the nature of sugar ester linkage. The ester groups on equatorial secondary hydroxyl are more readily hydrolyzed than those on axial secondary hydroxyl which in turn are more readily hydrolyzed than sulfate ester groups on primary hydroxyl groups [1]. Compared to these *O*-sulfate ester groups, the *N*-sulfate (sulfoamino) groups are much more acid labile [2].

The differential rate of hydrolysis of sugar sulfates has been used to distinguish between *N*-sulfate and *O*-sulfate [2] as well as to obtain information on the position of attachment of *O*-sulfate (for example, 4-sulfate vs. 6-sulfate) groups [3]. At best, such determination can be expected to give only tentative

Abbreviations: CPG column, controlled-pore glass beads column.

information, since when two or more types of sulfate groups co-exist in a molecule the rate of hydrolysis determined would be a composite of the different reaction rates.

In studies characterizing glycosaminoglycans synthesized by cultured cells, the release of inorganic sulfate on mild acid hydrolysis has been used as evidence for the presence of *N*-sulfate, and to calculate ratio of *N*- and *O*-sulfates in the molecule [4—7]. During our studies on the sulfated polysaccharides associated with the nuclei of mammalian cells, it was noticed that considerable release of inorganic sulfate occurred on mild acid hydrolysis of a fraction devoid of *N*-sulfate groups [8]. This finding prompted a re-examination of the effect of mild acid hydrolysis of some standard glycosaminoglycans, the results of which are reported in this publication.

## Experimental

Heparin sodium salt was from Sigma. Vitreous humor hyaluronic acid was purchased from Worthington. Protein polysaccharide light fragments (PPL) and chondroitin 4-sulfate were isolated as described [9]. Octa-, tetra- and disaccharides of chondroitin sulfate were prepared by hydrolysis of chondroitin sulfate [10].

A high molecular weight chondroitin sulfate was isolated from the spent media, whole cells and nuclei of B16 mouse melanoma cells grown in the presence of [$^3$H]glucosamine and $Na_2{}^{35}SO_4$ as described previously [8,11].

Hydrolyses of polysaccharide samples were done in capillary tubes prepared from disposable Pasteur pipettes. The sample (0.5—2.0 mg) was dissolved in 50—200 $\mu$l water, an equal volume of 0.08 M HCl was added and the tube sealed. Hydrolysis was carried out in a boiling water bath for 30 or 90 min. The capillary tubes were cooled, centrifuged to collect all the solution to the bottom and kept frozen until analyzed on columns. The samples were thawed, neutralized by the addition of NaOH and fractionated on a controlled-pore glass (CPG) or BioGel P2 column or used for paper electrophoresis. Controlled-pore glass beads (CPG 10-240 80/120 mesh) were treated with polyethylene glycol and packed in columns as a slurry with application of constant vibration. The columns were equilibrated with 0.5 M $CaCl_2$ and eluted with the same solution. BioGel P2 column (0.9 × 70 cm) was equilibrated and eluted with 0.1 M pyridine acetate. Rates of flow of both columns were maintained constant at 30 ml /h by means of a Milton Roy minipump in order to obtain reproducible fractionations. Fractions of about 1 ml were collected using an ISCO fraction collector equipped with a drop counter.

Fractions were analyzed for uronic acid by the orcinol reaction [12] or for radioactivity by scintillation counting on an Intertechnique Model SL36 spectrometer using 1 ml of aqueous sample and 10 ml of the counting liquid containing xylene and Triton X-114 [13].

## Results and Discussion

The results of paper electrophoresis before and after mild acid hydrolysis of double lableed ($^3$H and $^{35}$S) chondroitin sulfate isolated from cultured B16

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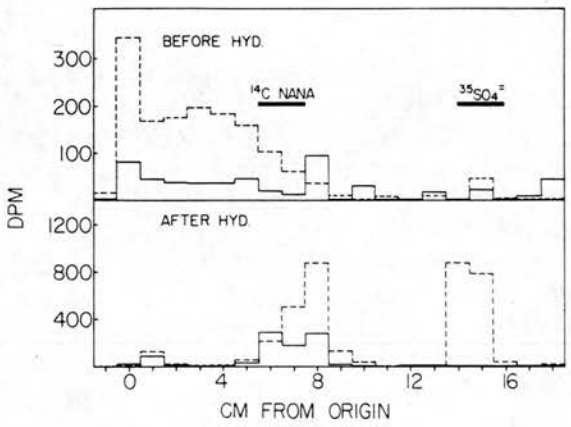


Fig. 1. Paper electrophoresis of mouse melanoma chondroitin sulfate before (upper) and after (lower) hydrolysis with 0.04 M HCl, 100°C for 90 min. Electrophoresis was done on Whatman No. 3 paper in 0.5 M pyridine acetic acid buffer, pH 5.0, at 10 V/cm for 2—3 h. Positions of markers *N*-[$^{14}$C]-acetylneuraminic acid ([$^{14}$C]NANA) and [$^{35}$S]sulfate were determined by scanning 1.5 inch wide strips on Packard Model 7201 radiochromatogram scanner. Tritium activity (solid line) and $^{35}$S activity (broken line) of samples were detected by extracting 1 cm strips and counting.

mouse melanoma cells is illustrated in Fig. 1. After hydrolysis with 0.04 M HCl at 100°C for 90 min, about 45% of the $^{35}$S radioactivity migrated as inorganic sulfate. This was surprising since this fraction has been characterized as having only *O*-sulfate residues by its complete susceptibility to chondroitinase AC and chondro-4-sulfatase [8]. The resistance of this component to $HNO_2$ treatment,

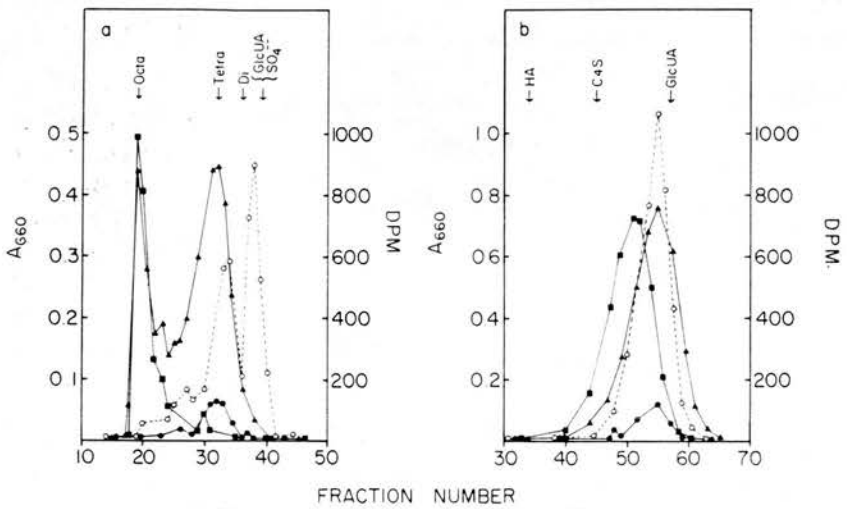


Fig. 2. Fractionation of mouse melanoma chondroitin sulfate (○------○, $^{35}$S and ●———●, $^{3}$H) and cartilage chondroitin sulfate (▲———▲) after hydrolysis with 0.04 M HCl, 100°C for 90 min. The elution of cartilage chondroitin sulfate after hydrolysis for 30 min (■———■) is also illustrated. Chromatography on BioGel P2 column (a) and on controlled-pore glass (CPG) column (b). The peak elution positions of marker saccharides (HA, vitreous humor hyaluronic acid; C4S, porcine rib cartilage chondroitin 4-sulfate; GlcUA, glucuronic acid; $SO_4^{2-}$, inorganic sulfate; octa-, tetra- and disaccharides, respectively, from chondroitin sulfate) are indicated by the arrows.

0.3 M $HNO_2$ at room temperature for 80 min, further confirmed the absence of *N*-sulfate residues. It was also apparent in the above experiment that in addition to the release of *O*-sulfate, the hydrolysis degraded the molecule to oligosaccharides. The results of chromatography of the acid hydrolysis products on BioGel P2 column (Fig. 2a) confirmed this finding. About 42% of the $^{35}S$ eluted in the position of inorganic sulfate while most of the remaining $^{35}S$ activity and all of the $^{3}H$ activity eluted in the area of chondroitin tetrasaccharide. The elution profiles of cartilage chondroitin sulfate hydrolyzed for 30 and 90 min are also shown in Fig. 2a. It is clear that after 90 min hydrolysis the major portion of this polysaccharide is degraded to fragments eluting in the position of chondroitin 4-sulfate tetrasaccharides. The balance eluting at the void volume are octasaccharide size or larger. The elution pattern on CPG columns of cartilage and melanoma chondroitin sulfates after acid hydrolysis further illustrates the degradation of these molecules (Fig. 2b). Whereas, the unhydrolyzed melanoma chondroitin sulfate co-elutes in the void volume with vitreous humor hyaluronic acid, the hydrolysis products are eluted entirely in the low molecular weight region of the column. When hydrolyzed for only 30 min, the extent of degradation of cartilage chondroitin sulfate is less as illustrated by the peak elution position at fraction 51 (Fig. 2b).

The elution profiles of hyaluronic acid and heparin after mild acid hydrolysis for 90 min on BioGel P2 and CPG columns are shown in Fig. 3. The results suggest that hyaluronic acid is completely degraded to fragments of octasaccharides or larger. In the case of heparin, the degradation appears to be minimal; before hydrolysis heparin eluted from CPG column in a very broad peak with maximum at fraction 47 and after hydrolysis the peak maximum shifted to fractions 49—50 (Fig. 3b).

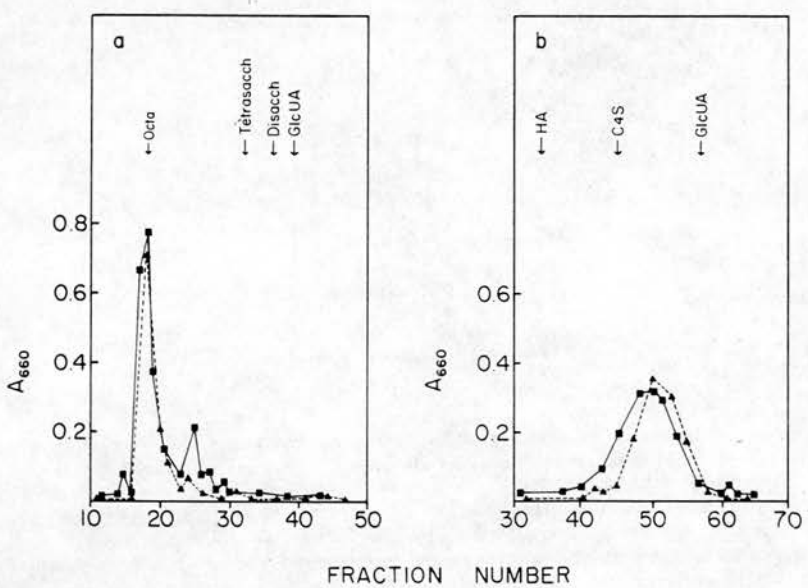


Fig. 3. Chromatography of hyaluronic acid (▲- - - - -▲) and heparin (■————■) after hydrolysis with 0.04 M HCl, 100°C for 90 min on a, BioGel P2; b, CPG columns. Abbreviations are as in Fig. 2.

Cartilage proteoglycan light fraction (PPL) when hydrolyzed for 90 min and chromatographed on CPG and BioGel P2 columns showed extensive degradation of the polysaccharide chains.

Heparan sulfate appears to be a ubiquitous component of the mammalian cell surfaces and has been implicated in control of cell growth [11,14,15]. Labeling with [$^{35}$S]sulfate and chromatographic techniques are widely used to facilitate the identification and characterization of sulfated polysaccharides synthesized by mammalian cells in culture. One of the criteria used to distinguish the *N*-sulfate heparan sulfate from other *O*-sulfated polysaccharides (chondrotin sulfate, dermatan sulfate, keratan sulfate) is the ease of release of sulfate from the former on mild acid treatment. It is clear from our results that this distinction is unreliable because of the unexpected ease of hydrolysis of *O*-sulfate.

The degradation of glycosaminoglycans by the mild acid hydrolysis is of interest and is in general agreement with the findings of Cifonelli [16]. The glycosaminoglycans (hyaluronic acid, chondroitin sulfates and chondroproteoglycan) containing *N*-acetylhexosamines were all degraded to oligosaccharides in the range of tetra- to octasaccharides. However, heparin having mostly *N*-sulfated hexosamines did not undergo detectable degradation under these conditions. This may be related to the very rapid cleavage of the *N*-sulfate bonds and the resistance to further hydrolysis of the generated hexosaminidic bond.

## Acknowledgement

This work was supported in part by Grants CA 15483 and 17686 from the U.S. Public Health Service.

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Reprinted from

*Carbohydrate Research*, 57 (1977) 173–188


# CHARACTERIZATION OF THE CHONDROITIN SULFATE PRODUCED BY B16 MOUSE MELANOMA CELLS*

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(Received December 16th, 1976; accepted for publication, February 8th, 1977)

## ABSTRACT

The mucopolysaccharides produced by B16 mouse melanoma cells have been isolated in milligram quantities from the spent media in which the cells were grown in the presence of 2-amino-2-deoxy-D-glucose-*t* and [$^{35}$S]-sulfate. The mucopolysaccharides obtained by precipitation with cetylpyridinium chloride from the Pronase digest of the media were further purified by gel filtration, ion-exchange chromatography, and treatment with nucleases. The major components were identified as chondroitin-4-sulfates by identification of the hexosamine as 2-amino-2-deoxy-D-galactose, and by digestibility with hyaluronidases, chondroitinase AC, and chondro-4-sulfatase. The o.r.d. curve and i.r. spectra of these components also confirmed their similarity to chondroitin-4-sulfate from cartilage. The molecular weight of the polysaccharide chains was estimated to be in the range 90,000–120,000 by sedimentation equilibrium analysis.

## INTRODUCTION

Mucopolysaccharides are associated with the surface of a wide variety of cultured normal and cancer cells[1,2]. They are thought to play a role in a variety of cell properties such as adhesion, contact inhibition, masking of receptors, and interaction with antigens. In addition, due to their high negative charge and their large size, they are also likely candidates for regulating macromolecule and cation access to the cell plasma membrane. This class of macromolecule is also associated with the nuclei[3–5], mitochondria[6], and plasma membrane[7]. Neither the nature of the association of mucopolysaccharides with these cell organelles nor their function is clearly understood.

A prior study of the complex saccharides produced by B16 mouse melanoma cells[8] showed the presence, both in the cells and spent media, of a family of chon-

*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S.

droitin sulfates having properties distinct from those of typical chondroitin sulfates of cartilage. This class of polymer was also the major mucopolysaccharide associated with the nucleus of these cells[3].

We now report on the chemical and physical characterization of the chondroitin sulfates produced by B16 mouse melanoma cells in culture.

## EXPERIMENTAL

*Materials.* — Fetal calf serum and antibiotic solution were obtained from Flow Laboratories, Rockville, MD, and other components for culture media from Grand Island Biological Company, Grand Island, N.Y. Pronase CB was obtained from Calbiochem; controlled-pore glass beads (CPG 10-240) and glyceryl-CPG 240 were from Electronucleonics, Fairfield, NJ; Sepharose 4B-200 and diethylaminoethyl (DEAE) Sephadex were from Sigma; Chondroitinases ACII (EC 4.2.2.5) and ABC (EC 4.2.2.4) and chondro-4-sulfatase (EC 3.1.6.9) were from Miles Laboratories; testicular hyaluronidase (EC 3.2.1.35) (400 units per mg) was from Worthington, Inc.; bacterial hyaluronidase (EC 4.2.99.1) from Organon; and leech hyaluronidase (EC 3.2.1.36) from Biotrics, Arlington, MA. Heparitinase was isolated from an adapted *Flavobacterium heparinum* strain provided by Dr. A. Linker[9,10]. Bovine pancreas deoxyribonuclease DN-EP (EC 3.1.4.5) was from Sigma, and bovine pancreas ribonuclease (EC 2.7.7.16) from Boehringer.

Vitreous humor hyaluronic acid was obtained from Worthington; proteoglycan and chondroitin-4-sulfate were isolated from pig-rib cartilage; and reference heparan sulfate was a gift from Dr. A. Linker. Streptococcal hyaluronic acid was a gift from Dr. Karl Meyer.

*Column chromatography.* — Glyceryl CPG-240 or CPG 10-240 beads (80–120 mesh) treated with polyethylene glycol were packed in silicone-coated columns with constant vibration according to the instructions of the manufacturers. The columns were equilibrated with 0.5M KCl and eluted with the same solution; a pump was used to maintain a constant flow rate of 30 ml/h. Columns (0.9 × 70 cm) of Biogel P2 were equilibrated and eluted with 0.1M pyridine acetate (pH 5.0). Sepharose 4B and Sephadex G200 were packed in columns (2 × 60 cm) and equilibrated with 50 mM Tris-HCl buffer (pH 8.0).

DEAE Sephadex A-25-120 was swollen in M sodium acetate for 48 h, filtered off, and washed with 0.01M pyridine acetate buffer (pH 5.1) on a Buchner funnel until the filtrate gave a negative test for chloride. A suspension of the beads in the same buffer was packed in a column (13 × 1 cm).

Cellulose acetate electrophoresis was performed in a Beckman R-101 microzone electrophoresis cell with 0.2M calcium acetate (pH 7.0) at 5 mA for 3 h, or pyridine–formic acid buffer (0.1M in formic acid, pH 3.0) at 10 mA for 20 min. Staining with Alcian Blue (0.1% in 0.5% acetic acid) was used for detecting acidic components (mucopolysaccharides and mucin-type sialoglycoproteins).

Unless specified otherwise, all dialysis was performed at 4° in the presence of toluene and chloroform.

Hexosamine determinations on isotopically labeled components were carried out, after acid hydrolysis, by ion-exchange chromatography on an automated amino acid analyzer; the stream-splitting technique described earlier[8,11] was used. The samples were hydrolyzed with 6M HCl at 110° *in vacuo* for 24 h; on the basis of control experiments, a correction for the destruction of 28.5% of 2-amino-2-deoxyglucose and 25.5% of 2-amino-2-deoxygalactose was applied. G.l.c. identification of neutral sugars was performed, following hydrolysis as described in the text, by the alditol acetate procedure[12]. A Packard gas–liquid chromatograph was used[13] with a glass column (6 ft × 0.125 in.) of 3% of ECNSS-M on Gas Chrom Q (100–120 mesh). Uronic acids were identified as the trimethylsilyl derivatives[14] [15% of Apiezon M on Gas Chrom CLZ (80–100 mesh)]. Marker polysaccharides run on gel columns were assayed by the orcinol reaction[15]. 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[16]. Efficiencies for $^{3}H$ and $^{35}S$ were ~16 and ~47%, respectively, with a crossover of ~13–14% of $^{35}S$ into the $^{3}H$ channel. The citrate buffer fractions (4 ml) from the amino acid analyzer were mixed with 10 ml of 3a40 counting fluid (Research Products International Corporation) and counted. The efficiency for tritium in this system was 15%, using the same settings for minimal crossover of $^{35}S$ 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 was mixed and counted.

*Cell cultures.* — B16 mouse melanoma cell lines (B16C2 and B16C3) and an amelanotic clone were grown in 16-oz prescription bottles and subcultured at confluency by suspension with 0.02% of EGTA [ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid] in calcium- and magnesium-free phosphate buffered saline. The cells were cultured in Eagle's medium[17] containing twice the recommended concentration of vitamins and supplemented with non-essential amino acids (each at 0.1mM), sodium pyruvate (mM), and 10% heat-inactivated (56° for 30 min) fetal-calf serum. Cultures were routinely tested for bacterial contamination by the use of thioglycolate and tryptose phosphate broth[18], and for yeast and mold contamination with Sabouraud's medium[19]. The spent media at each medium change, and at subculturing, were decanted, centrifuged to remove floating cells, and stored in the freezer. We are grateful to Mr. John Banks for help in cell culture and for collecting media used in these studies. Spent medium containing labeled complex saccharides was prepared by culturing the cells for 48 h prior to harvest in medium containing (per ml) 10 μCi of 2-amino-2-deoxy-D-glucose-*t* (New England Nuclear, 755 mCi per mmol) and 50 μCi of $Na_2{}^{35}SO_4$ (New England Nuclear, 755 mCi per mmol).

*Treatment of media and of fetal-calf serum with Pronase and alkali.* — Pooled, frozen, spent media (~5 l, MI) was thawed and dialyzed at 4° against 0.9% NaCl for 2 days, and then against water for 5 days. The dialyzed material was concentrated by

ultrafiltration and lyophilization to ~600 ml and combined with 100 ml of dialyzed, labeled media. Calcium acetate was added (to 0.01M $Ca^{2+}$) and the pH was adjusted to 7.8 with 10M NaOH. Pronase (100 mg) was stirred in, toluene (2 ml) added, and the solution incubated in a stoppered flask at 40° 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 the addition of NaOH. The digest was centrifuged, the residue discarded, and the supernatant solution dialyzed against 0.9% NaCl overnight, and then against deionized water for 4 days. The contents of the dialysis bag were adjusted to pH 12.5 and, after 18 h at 20–25°, dialyzed for 5 days against several changes of deionized water. A second batch of spent media MII (3 l) was worked up in an identical manner, except that the treatment at pH 12.5 for 18 h was omitted.

As a control, heat-inactivated fetal-calf serum (300 ml) was digested with Pronase as described above for the second batch of media, and then dialyzed against 0.9% NaCl followed by deionized water for a total of 5 days.

*Precipitation of digested media and serum with cetylpyridinium chloride.* — The Pronase- and alkali-treated material was adjusted to 0.03M in NaCl and 0.15% in cetylpyridinium chloride (CPC). After 48 h at room temperature, the precipitate was collected by centrifugation (15,000 *g*, 30 min) and washed three times with 0.15% of CPC in 0.03M NaCl by resuspension and centrifugation. CPC was added to the supernatant solution and washings to a final concentration of 1%. The small amount of precipitate formed was collected and washed as above. The precipitates were combined and fractionally extracted by stirring with 0.2, 0.4, 0.6, 1.0, 1.2, and 2.0M NaCl, utilizing $1 \times 20$ ml and $3 \times 10$ ml for each extraction; at least one of the extractions at each molarity was done overnight. The CPC in the extracts was removed by dialysis at 40–45° against 2M NaCl followed by distilled water at 4°; the solutions were then filtered and lyophilized.

*Enzyme digestions.* — The labeled samples (10,000–25,000 d.p.m., $^3H$) were mixed with 500 μg of carrier mucopolysaccharide (hyaluronic acid, chondroitin sulfate, or heparan sulfate) and digested with hyaluronidase, chondroitinase, or heparitinase as described below. The carrier mucopolysaccharides were completely degraded under the conditions employed. Testicular hyaluronidase digestion was performed in 700 μl of 0.1M sodium acetate buffer (pH 5.0) and 8mM EDTA in the presence of toluene and chloroform at 37° for 24 h, using 80 units of enzyme. Bacterial hyaluronidase digestion was performed in 0.1M sodium acetate buffer (pH 5.0) at 37° for 26 h, using 150 units of enzyme in a total volume of 150 μl. Leech hyaluronidase digestion was carried out by dissolving the sample in 100 μl of McIlvaine's buffer (pH 5.6) and incubating at 37° for 6 h with the addition of 125 μg of enzyme in 25 μl of buffer at 0, 1, and 2 h. Heparitinase digestions[9] were done in 300 μl of 0.1M sodium acetate buffer (pH 7.0) containing mM calcium acetate at 43° for 24 h. Chondroitinase and chondrosulfatase digestions were performed according to the method of Saito *et al.*[20].

Nitrous acid degradation was done by treating the sample in 100 μl of water with 20 μl of 3M $NaNO_2$ and 20 μl of glacial acetic acid at room temperature for

80 min. Excess of nitrite was destroyed by addition of 50 $\mu$l of 3M glycine and, after 60 min at room temperature, the product was lyophilized.

The molecular weights ($\overline{M}_w$) of standard saccharides and unknown samples were determined by sedimentation equilibrium on a Beckman Model E analytical ultracentrifuge by using Rayleigh interference optics. Samples dissolved in an electrolyte, usually 0.5M NaCl, were run at 25° in a three-channel cell at three different concentrations[21]. The fringe patterns were recorded photographically, and the data were read and analyzed directly by a computer-operated system[22].

O.r.d. data were obtained on a Cary Model 60 Spectropolarimeter with samples dissolved in M NaCl; a 10-mm cell was used. Infrared spectra (KBr pellets) were recorded on a Perkin–Elmer 267 spectrophotometer.

## RESULTS

Table I presents the results of CPC fractionation of the Pronase digest of the two lots of spent media (MI, 5 l; and MII, 3 l) and of fetal-calf serum (S). Fractions MI 0.2 and MII 0.2, which were eluted from the CPC precipitate with 0.2M NaCl, consisted mainly of a mucin-type sialoglycopeptide which has been characterized[11].

*0.4M NaCl Fractions.* — MI 0.4 (5.5 mg) was fractionated on a CPG column (1.5 × 115 cm), as illustrated in Fig. 1 (middle). The fractions were combined as indicated, dialyzed, and lyophilized to yield MI 0.4a (1.8 mg) and MI 0.4b (3.2 mg). The distribution of [$^3$H]-activity in the hexosamine component of these fractions is given in Table II. The major portion of MI 0.4b was identified as a mucin-type sialoglycopeptide similar to that present in the 0.2M NaCl eluates. Cellulose acetate electrophoresis of MI 0.4a was done in the two buffer systems; the result in calcium acetate buffer is shown in Fig. 2. Two components were detected with Alcian Blue, but the radioactivity was associated entirely with the spot having the higher mobility.

Separation of the labeled component from the unlabeled contaminant was achieved by chromatography of MI 0.4a on a column (1 × 13 cm) of DEAE Sephadex. After loading of the sample, the column was eluted with a linear gradient of 0.01 → 1.0M pyridine acetate (pH 5.1). Fractions (180 × 1 ml) were collected and aliquots assayed for radioactivity; none was detected in these fractions. The labeled component was recovered from the column by elution with 2M NaCl, dialysis, and lyophilization (MI 0.4a2). The fractions (1–180) were also combined, concentrated, and recovered (MI 0.4a1). The results of cellulose acetate electrophoresis of these two fractions are illustrated in Fig. 2. Fraction (MI 0.4a2) gave only one spot and was free of the unlabeled component (MI 0.4a1). Component MI 0.4a1 was hydrolyzed, and analyzed on a Beckman model 121 amino acid analyzer (60-cm column). In addition to amino acids, both 2-amino-2-deoxyglucose and 2-amino-2-deoxygalactose were present in approximately equal amounts; the component was apparently derived from serum and was not further examined.

The molecular weight of MI 0.4a2 was determined by equilibrium sedimentation to be 92,500; a partial specific volume ($\bar{v}$) for chondroitin sulfate[23] of 0.57 was used

TABLE 1

RESULTS OF CPC PRECIPITATION AND SALT ELUTION OF PRONASE-DIGESTED SPENT MEDIA AND FETAL-CALF SERUM

| *Fraction*[a] | *Media I (MI; 5 l)* | | | *Media II (MII; 3 l)* | | | *Fetal-calf serum (S; 300 ml)* |
|---|---|---|---|---|---|---|---|
| | *Weight (mg)* | $^{3}H$ *(d.p.m.* $\times 10^{-6}$*)* | $^{35}S$ *(d.p.m.* $\times 10^{-4}$*)* | *Weight (mg)* | $^{3}H$ *(d.p.m.* $\times 10^{-6}$*)* | $^{35}S$ *(d.p.m.* $\times 10^{-4}$*)* | *Weight (mg)* |
| 0.2M NaCl | 7.4 | 2.28 | 0.47 | 1.9 | 0.64 | 0.09 | 0.7 |
| 0.4M NaCl | 7.8[b] | 0.40 | 1.48 | 7.1 | 2.40 | 1.12 | 1.1 |
| 0.8M NaCl | 12.1 | 1.93 | 18.83 | 6.9 | 0.66 | 20.00 | 2.4 |
| 1.2M NaCl | 2.2 | 0.69 | 0.81 | 0.5 | 0.33 | 8.40 | 0.3 |
| 2.0M NaCl | 2.0 | 0.06 | 0.80 | 0.9 | 0.02 | 2.20 | 3.8 |

[a]These fractions are referred to as MI0.4, MII0.4, etc., in the text. [b]Some of this fraction (MI0.4) was lost during work-up of the extract.

for the calculation (Table II). The sample recovered after molecular weight determination was used for o.r.d. measurements. The results are shown in Fig. 3. The sample was then dialyzed against water, KBr (300 mg) was added, and the solution was lyophilized. The resulting powder was dried in a desiccator over $P_2O_5$, and pressed into a pellet, and the i.r. spectrum was recorded (Fig. 4).

O.r.d. measurements and i.r. spectra were also done on cartilage proteoglycan, hyaluronic acid, and cartilage chondroitin-4-sulfate standards.

Chromatography of the fraction MI 0.4a2 before and after treatment with alkaline borohydride (M $NaBH_4$–0.1M NaOH, 37°, 72 h) gave the results shown in Fig. 5. There was very little change in molecular size on treatment with alkaline borohydride.

Fraction MII 0.4 was also separated into MII 0.4a and MII 0.4b on a CPG column (Fig. 1). MII 0.4a (1.69 mg), consisting of mucopolysaccharide of high molecular weight, was further purified on a column of Sepharose 4B as described

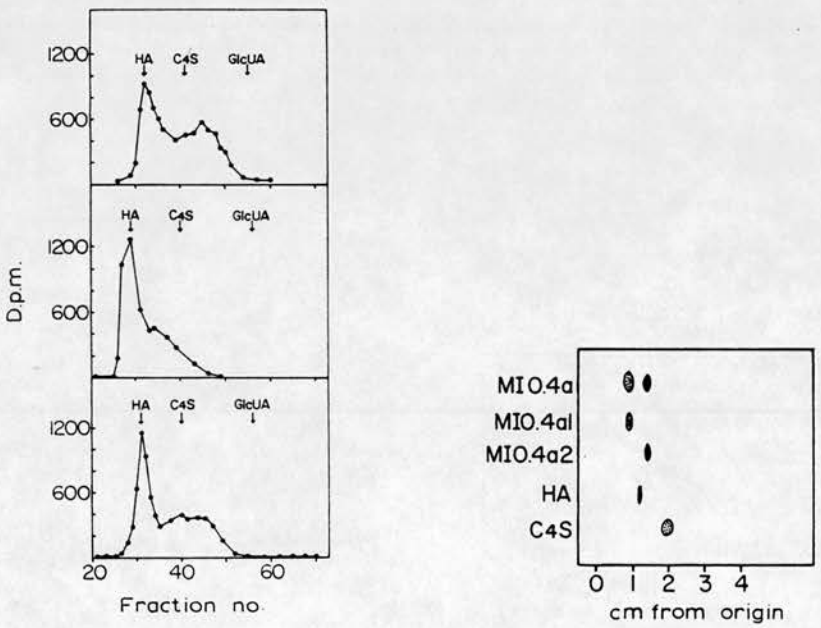



Fig. 1. Fractionation of 0.4M or 0.8M NaCl fractions on a column (1.5 × 115 cm) of CPG 10-240 beads; fractions (3 ml) were collected and analyzed for radioactivity ($^3H$). The peak-elution position of marker saccharides (HA, vitreous humor hyaluronic acid; C4S, porcine rib-cartilage chondroitin-4-sulfate, and GlcUA, glucuronic acid) are indicated by the arrows. MI0.8 (upper); fractions 29–38 and 39–52 were separately combined and recovered to yield MI0.8a and MI0.8b, respectively. MI0.4 (middle); fractions 26–32 and 33–45 were recovered to yield MI0.4a and MI0.4b, respectively. MII0.8 (lower); fraction 27–33 and 34–52 were combined and recovered to yield MII0.8a and MII0.8b, respectively.

Fig. 2. Cellulose acetate electrophoresis in calcium acetate buffer of MI0.4a before and after (MI0.4a1 and MI0.4a2) fractionation on a column of DEAE Sephadex; details are given in the text. Shaded spots stained more intensely with Alcian Blue than the dotted spots.

TABLE II

ANALYTICAL DATA FOR VARIOUS MEDIA SUBFRACTIONS

| *Fraction* | *Weight (mg)* | *GlcN*[a] | *GalN*[a] | *Mol. Wt.*[b] |
|---|---|---|---|---|
| MI0.4a | 1.80 | 2.8 | 97.2 | 47,400 |
| MI0.4b | 3.21 | 8.8 | 91.2 | ~15,000 |
| MI0.4a1[c] | 0.42 | n.d.[d] | n.d. | n.d. |
| MI0.4a2[c] | 1.17 | 0 | 100.0 | 92,500[e] |
| MII0.4a[f] | 1.69 | n.d. | n.d. | 120,600 |
| MI0.8a | 1.38 | 5.8 | 94.2 | 80,000 |
| MI0.8b | 1.53 | 71.5 | 29.5 | ~15,000 |
| MII0.8a[g] | 0.80 | 3.5 | 96.5 | 121,000 |
| MII0.8b | 1.40 | 67.5 | 32.5 | ~15,000 |

[a]The hexosamine values are as percentages of the tritium label found for 2-amino-2-deoxy-glucose and -galactose. [b]Based on the behavior of the labeled components relative to standard mucopolysaccharides during chromatography on CPG (MI0.4b, MI0.8b, and MII0.8b) or by sedimentation equilibrium analysis (others). [c]Obtained by fractionation of MI0.4a on DEAE Sephadex; see Results for details. [d]n.d., Not determined. [e]Average of two separate determinations (88,000 and 97,000). [f]Determined after purification on Sepharose 4B. [g]Determined after treatment with DNase, RNase, and Pronase, and purification by chromatography on Sephadex G200 and Sepharose 4B.

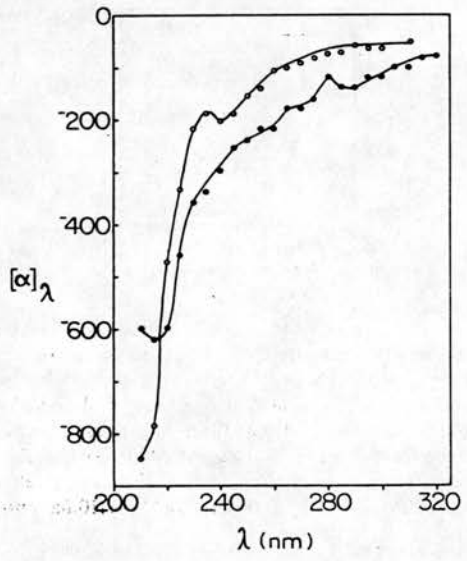



Fig. 3. O.r.d. curves for fractions of mouse melanoma chondroitin sulfate: —○—, MI0.4a2; and —●—, MI0.8a.

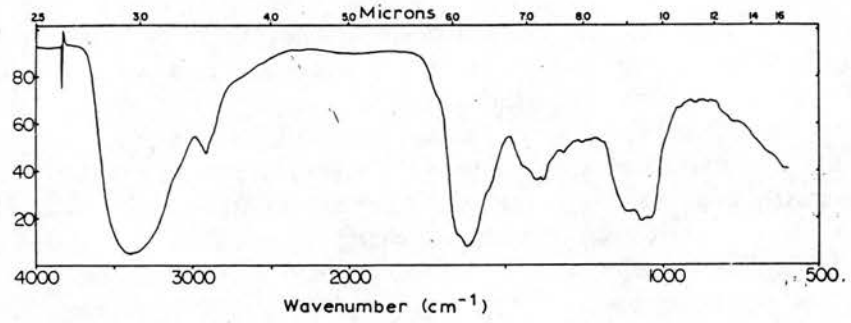



Fig. 4. Infrared spectrum for fraction MI0.4a2 of mouse melanoma chondroitin sulfate.

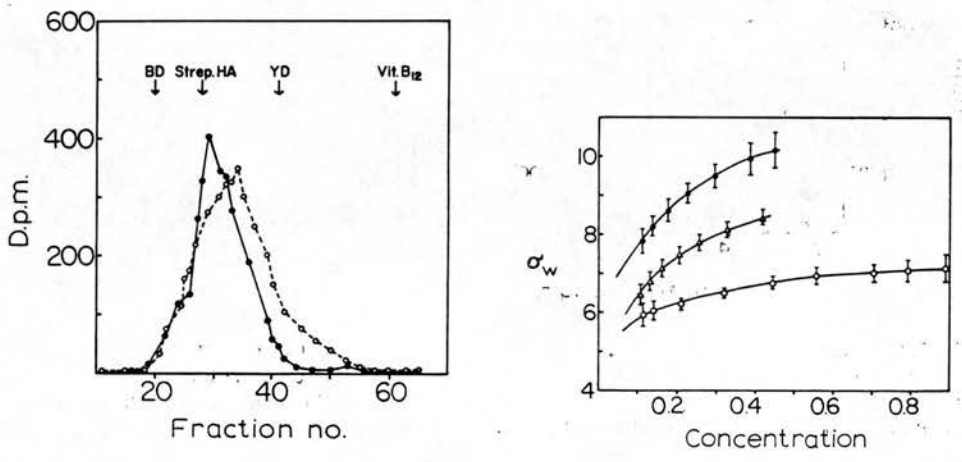



Fig. 5. Gel filtration of mouse melanoma fraction MI0.4a2 before (—●—) and after (—○—) treatment with M $NaBH_4$–0.1M NaOH (37° for 72 h) on a column of Sepharose 4B (2 × 60 cm). Elution positions of calibration standards are indicated by arrows: BD, blue dextran; YD, yellow dextran; Vit. $B_{12}$, vitamin $B_{12}$; and Strep. HA, streptococcal hyaluronic acid. The fractions were analyzed for $^3H$ activity.

Fig. 6. Molecular moments for fraction MII0.4a of mouse melanoma mucopolysaccharide in 0.5M NaCl: r.p.m. = 15,000; loading concentration channel A (●), 0.5 mg/ml; channel B (△), 0.25 mg/ml; and channel C (○), 0.125 mg/ml.

below for MII 0.8a, and the main peak was used for determination of molecular weight. A molecular weight ($\overline{M}_w$) of 120,600 ±5800 was estimated (Fig. 6); a two-species plot[24] showed the presence of aggregates up to trimers.

*0.8M NaCl Fractions.* — Fractionation on a CPG column gave the elution pattern shown in Fig. 1 (upper); MI 0.8a (1.38 mg) and MI 0.8b (1.53 mg) were obtained. Hexosamine analyses of these fractions are given in Table II. Cellulose acetate electrophoresis of MI 0.8a was done in two buffer systems. In pyridine formate buffer, the major spot moved slightly ahead of hyaluronic acid, and the second spot,

present only in trace amounts, had a mobility similar to that of heparan sulfate (Fig. 7). O.r.d. measurements were done on MI 0.8a (Fig. 3), which had $[\alpha]_{280}$ $-120°$; porcine rib-cartilage chondroitin sulfate has $[\alpha]_{280}$ $-219°$, and vitreous humor hyaluronic acid $-520°$. This fraction was digested with Pronase for 48 h at 40°, with two additions of the enzyme at 0 and 24 h. The gel-filtration pattern on CPG of the digested material was the same as that of the undigested fraction. This indicates that the molecule does not consist of several saccharide chains attached to a Pronase-susceptible peptide, in agreement with earlier observations on material isolated from the nuclei of melanoma cells[3].

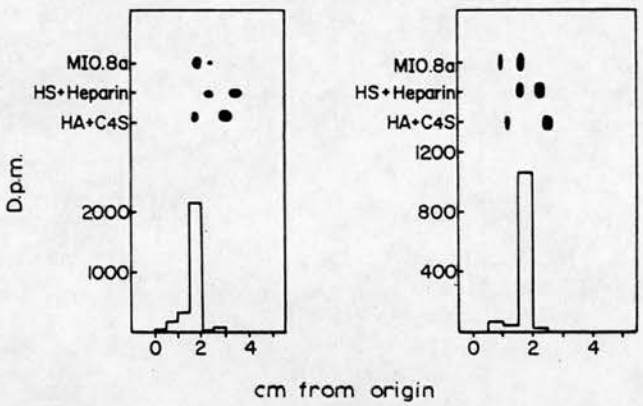



Fig. 7. Cellulose acetate electrophoresis of mucopolysaccharide fraction MI0.8a from mouse melanoma in pyridine acetate (left) and calcium acetate (right) buffers. The unknown sample was run in two lanes; after electrophoresis, one lane was assayed for radioactivity (see Methods) and the remainder of the strip was stained with Alcian Blue.

This fraction was digested with testicular hyaluronidase and chondroitinase AC, and the digests were examined by chromatography on CPG columns. The material was completely susceptible to both of these enzymes. The fraction was also susceptible to bacterial hyaluronidase, but the fragments obtained were larger than tetrasaccharides and disaccharides, as illustrated for the nuclear material[3]. These results are in agreement with a polysaccharide having 50% or less of the disaccharide repeating-units sulfated, more or less at random. Chondroitinase AC and chrondroitinase AC plus chondro-4-sulfatase digestion of this fraction, followed by paper chromatography as illustrated in Fig. 8, indicates that the sulfate ester group is mainly at position 4 of the 2-amino-2-deoxygalactose moiety.

In order to identify the uronic acid and neutral sugars by g.l.c., and the hexosamine by the ninhydrin-degradation method, this fraction was hydrolyzed under two different conditions and the products were separated as shown in Fig. 9. The uronic acid was identified as glucuronic acid; there was no evidence for the presence of iduronic acid, thereby eliminating the possibility of a chondroitin sulfate–dermatan sulfate co-polymer[25]. The results of the neutral sugar analysis were not very clear, as

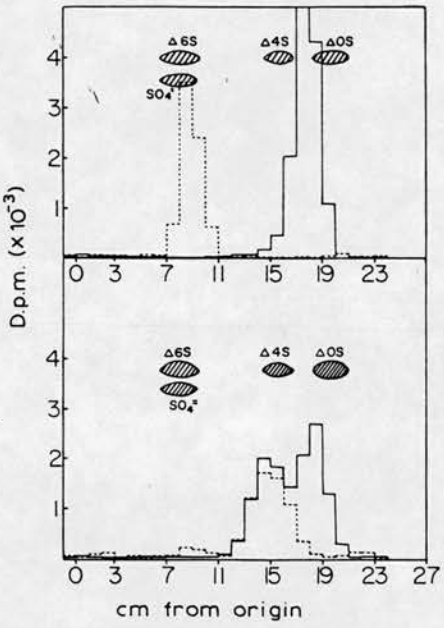
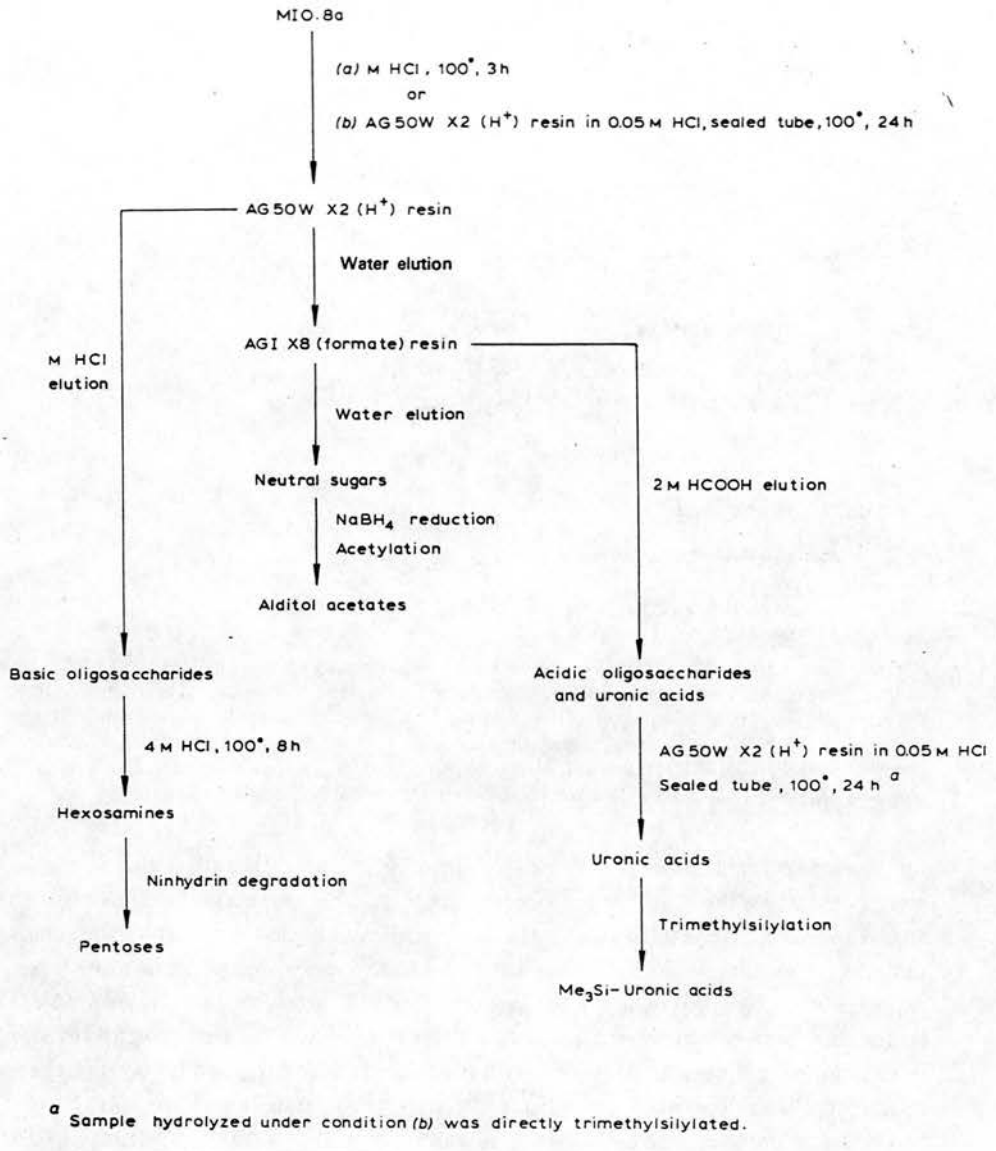



Fig. 8. Paper chromatography (1-butanol–acetic acid–M ammonium hydroxide, 2:2:1; 18 h) of chondroitinase AC (lower) and chondroitinase AC and chondro-4-sulfatase (upper) digests of fraction MI0.8a. The positions of reference compounds are indicated: Δ4S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose; Δ6S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔOS, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose.

there were several unidentifiable peaks. However, small peaks of xylose and galactose were detectable, but their stoichiometry could not be assessed; the presence of these sugars needs to be confirmed by independent methods. Polysaccharide chains of molecular weight ~120,000, if synthesized *via* glycosyl-serine initiation, would be expected to have only one xylose residue per 600 monosaccharide residues. Thus, larger amounts of samples are needed for quantitation of the neutral sugars. The hexosamine fraction was degraded with ninhydrin[26], and the products were examined by paper chromatography (ethyl acetate–pyridine–water, 9:3:2) with arabinose and lyxose as reference sugars. The labeled material had a mobility identical to that of lyxose, confirming the identity of the hexosamine as 2-amino-2-deoxygalactose; arabinose was not detected.

A major portion of MI 0.8b was susceptible to nitrous acid treatment. As 72% of the hexosamine in this fraction was 2-amino-2-deoxyglucose, it can be concluded that heparan sulfate is the major constituent of MI 0.8b. The remainder is apparently chondroitin sulfate[3] of molecular weight 10–15,000. When MI 0.8b was treated with heparitinase and chromatographed on CPG, ~60% of the $^{3}$H-radioactivity was eluted in the region of low molecular weight, a result in agreement with the nitrous acid degradation.




[a] Sample hydrolyzed under condition (b) was directly trimethylsilylated.

Fig. 9. Scheme for degradation and isolation of neutral sugars, uronic acids, and hexosamines from mouse melanoma fraction MI0.8a.

Fraction MII 0.8 was separated into fractions of higher and lower molecular weight on a CPG column (1.5 × 115 cm) (Fig. 1, lower). The fraction of higher molecular weight (MII 0.8a, 2.2 mg) was further purified as follows. It was dissolved in 50mM Tris–HCl buffer (pH 7.4) containing 10mM $Mg^{2+}$, and treated with deoxyribonuclease I and ribonuclease at 37° for 48 h. The product, recovered by dialysis and freeze-drying, was then dissolved in 50mM Tris–HCl buffer (pH 8.0) containing

10mM $Ca^{2+}$, and digested with Pronase at 40° for 3 days in the presence of toluene; enzyme was added at time 0 and at 24 h. The digest was chromatographed on a column of Sephadex G-220, and the peak containing all the radioactivity was recovered and chromatographed on a column of Sepharose 4B (Fig. 10). The component in the sharp portion of the peak (fractions 26–39) was recovered (0.72 mg). Cellulose acetate electrophoresis of this peak in calcium acetate buffer gave a single Alcian Blue-staining spot of mobility intermediate between that of hyaluronic acid and chondroitin-4-sulfate. The molecular weight of this purified material was estimated to be 121,000 by sedimentation equilibrium analysis; a two-species plot gave evidence for the presence of aggregates (trimers). Digestion of a portion of this fraction with leech hyaluronidase, with chromatography on a CPG column, gave the results shown in Fig. 11a. In parallel experiments, it was found, as expected, that the enzyme digested hyaluronic acid, but had no action on chrondroitin-4-sulfate from porcine rib-cartilage. The action of leech hyaluronidase on proteoglycan is illustrated in Fig. 11b. There is a slight change in the molecular size distribution, suggesting there may be a few leech hyaluronidase-susceptible bonds in proteoglycan.

*Serum fraction S 0.8.* — This fraction was also digested with deoxyribonuclease, ribonuclease, and finally Pronase, as described above. The digested sample was chromatographed on a column of Sepharose 4B, and the fractions (26–39) corresponding to the peak in Fig. 10 were combined, dialyzed, and lyophilized to yield 0.59 mg of material. Similarly, fractions 16–25 and 40–70 were also recovered, yielding 0.12 and 1.80 mg, respectively. Cellulose acetate electrophoresis of these fractions in pyridine acetate and calcium acetate buffers did not show any Alcian Blue-staining spots when spotted in the usual concentrations. When fifteen times the usual amount was subjected to electrophoresis, very diffuse areas were stained with Alcian Blue. Fractions 26–39 showed staining in an area of lower mobility than the cor-

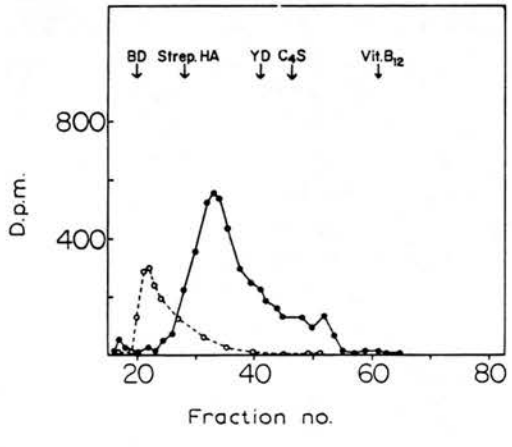



Fig. 10. Chromatography of MII0.8a (—●—) on a column (2 × 60 cm) of Sepharose 4B after treatment with deoxyribonuclease, ribonuclease, and Pronase. Elution pattern of vitreous humor hyaluronic acid (—○—) and the peak-elution positions of other standards (see Figs. 1 and 5) are shown.

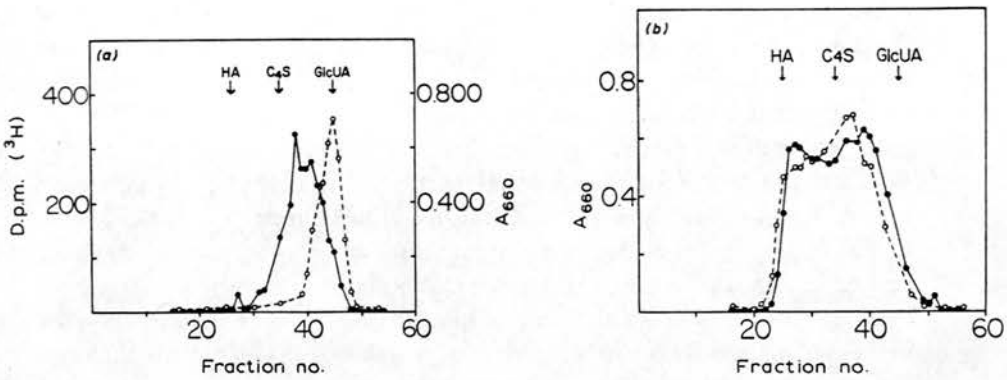



Fig. 11. (*a*) Chromatography of fraction MII0.8a (—●—) and hyaluronic acid (—○—) on a column (0.9 × 61 cm) of controlled-pore glass (CPG) 10-240 beads after digestion with leech hyaluronidase. Abbreviations are as in Fig. 1. Before treatment, both samples were eluted at the position indicated by HA. (*b*) Elution patterns of cartilage proteoglycan, before (—●—) and after (—○—) digestion with leech hyaluronidase, on the same CPG column.

responding fractions from MII 0.8a. Fractions 40–70 from serum, however, gave a streaky spot with the mobility of rib-cartilage chondroitin-4-sulfate.

## DISCUSSION

The purity of the labeled polyanionic components isolated from culture media was established by the criteria that the radioactivity and Alcian Blue-staining material co-migrated on cellulose acetate electrophoresis in two different buffer systems. Chemical characterization of the components is based mainly on the distribution of radioactivity in hexosamines, and on the susceptibility of the labeled component to enzymes. In addition, the charge properties, as determined by the elution from the cetylpyridinium chloride precipitate, and by the behavior on electrophoresis, confirm the identity of the components. The identification of the major mucopolysaccharide components as a family of chondroitin-4-sulfates of high molecular weight, and the minor components as heparan sulfate and chondroitin sulfates, is in agreement with previous results[3,8].

The susceptibility of the chondroitin sulfate of high molecular weight to leech hyaluronidase (Fig. 11) was surprising. This enzyme is an endo-glucuronidase, and is considered specific for linkages between 2-acetamido-2-deoxy-D-glucose and D-glucuronic acid[27]. It is possible that the melanoma chondroitin sulfate may have a few 2-acetamido-2-deoxy-D-glucose residues in the chain. Cleavage of only a few bonds in the molecule could cause the change in molecular size observed on CPG columns, whereas the presence of 2% of 2-amino-2-deoxy-D-glucose (3–4 residues) might not be detected by the analytical techniques described. There was insufficient material to further investigate this possibility.

Physical measurements were carried out on the chondroitin of high molecular weight, and the chondroitin-4-sulfate having ~50% sulfation, which eluted from the

CPC precipitate with 0.4 and 0.8M NaCl, respectively. Fig. 6 shows the results of a representative sedimentation-equilibrium analysis of these samples. The sample shows considerable polydispersity in molecular weights, as each concentration gave a different curve. The weight-average molecular weights ($\overline{M}_w$) calculated from several experiments were in the range of 80,000–120,000. The elution pattern of the labeled component from a column of Sepharose 4B (Figs. 5 and 10), in relation to streptococcal and vitreous humor hyaluronic acids, is in agreement with the above values[28]. The lower values of 80,000–95,000 may be due to the presence of contaminants of low molecular weight in these samples. For example, the sample MI 0.4a, which still had a serum component (MI 0.4a1) of low molecular weight as a contaminant, had $\overline{M}_w$ 47,000. The apparent polydispersity of the molecules could be partly due to associative interactions. A two-species plot indicated the presence of aggregates (monomer–trimer systems) of these polyanionic molecules, in agreement with earlier observations on cartilage proteoglycan[24]. It can be concluded that the molecular size of the melanoma chondroitin sulfate is in the range of 90,000–120,000, compared to 13,000 for the rib-cartilage product[24]. As the melanoma product was not susceptible to further Pronase digestion or to degradation with alkaline borohydride, it is more likely to be a single polysaccharide chain and not several smaller chains attached to a common peptide.

O.r.d. of the melanoma component (Fig. 3) was characteristic of mucopolysaccharides with a trough at 210–215 nm. The o.r.d. spectrum of chondroitin-4-sulfate chains from cartilage showed a trough at 220 nm, and that of vitreous humor hyaluronic acid at 215 nm. The infrared spectrum of MI 0.4a2 resembled that of cartilage chondroitin-4-sulfate, except for the absence of absorption in the sulfate region between 1230–1250 $cm^{-1}$. The lack of sulfate absorption is expected, as the fraction eluting with 0.4M NaCl from the CPC precipitate is low in sulfate. The $^{35}S/^{3}H$ ratio in MI 0.4 is 0.037, compared to 0.098 for MI 0.8 (Table I). The infrared spectra of hyaluronic acid and of cartilage proteoglycan were distinguishable from that of the melanoma component.

It has been reported that fetal-calf serum contains acid mucopolysaccharides in addition to large quantities of glycoproteins[29]. The chondroitin sulfate of high molecular weight identified above was metabolically labeled with 2-amino-2-deoxy-D-glucose-*t* and $^{35}SO_4^{2-}$ as illustrated, for example, by the production of disaccharides labeled with $^{3}H$ and $^{35}S$ on digestion of MI 0.8a with chondroitinase AC (Fig. 8). This, together with the fact that it was also isolated from the cell pellet[8] and nuclei[3], is inconsistent with its being a serum component. The possibility of a serum contaminant co-purifying with the melanoma chondroitin sulfate and leading to misinterpretation of the physical data was tested by carrying out a control experiment with fetal-calf serum. The fractions eluting with 0.4 and 0.8M NaCl were isolated from fetal-calf serum by procedures identical to those used for spent media. However, on fractionation of the 0.8M fraction on Sepharose 4B, the major portion was eluted in the region of low molecular size and behaved in cellulose acetate electrophoresis like cartilage chondroitin sulfate. The material (~0.6 mg) eluted in the same region

as the melanoma chondroitin sulfate may not be a polyanionic component because, even at fifteen times the usual concentration, it failed to stain with Alcian Blue when examined by cellulose acetate electrophoresis.

ACKNOWLEDGMENTS

We are grateful to Ms. Debora Breindel and Mr. Jeffrey Kemper for technical assistance, Mr. Mord Moore for hexosamine analysis, and Mr. John Banks for help with cell culture. We also thank Dr. Dennis Roark and his assistants (Messrs. George Keller, Karl Matter, and Richard Engle) for the equilibrium ultracentrifugation analysis. This work was supported by Grants CA15483 and CA17686 from the U.S. Public Health Service.

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[Reprinted from Biochemistry, (1977) 16, 4426]


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

Veerasingham P. Bhavanandan,* Junji Umemoto, John R. Banks, and Eugene A. Davidson

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, $\beta$-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, $\alpha$-*N*-acetylgalactosaminidase, and $\beta$-hexosaminidase (all from *Charonia lampis*) were purchased from Miles Laboratories; Tos-PheCH$_2$Cl-trypsin was obtained from Worthington Biochemical Corp. *endo*-$\alpha$-*N*-Acetylgalactosaminidase and $\beta$-hexosaminidase from *Diplococcus pneumoniae* were isolated as described (Bhavanandan et al., 1976; Umemoto et al., 1977). Jack bean $\beta$-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.

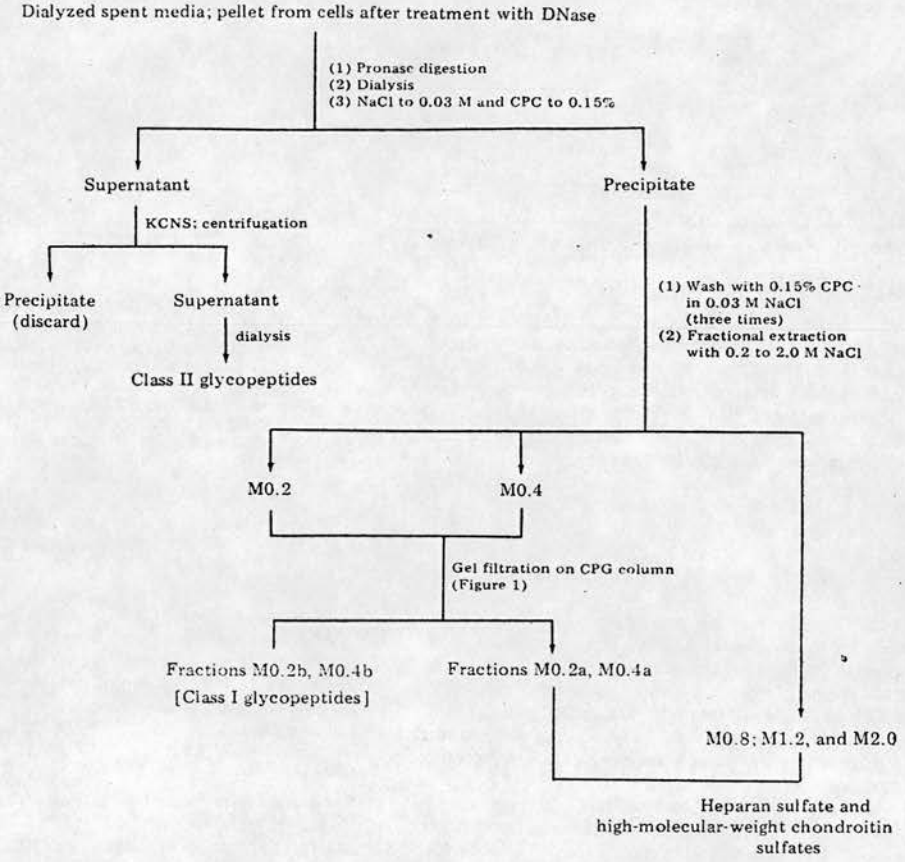
*Digestion with Glycosidases. Vibrio cholerae* neuraminidase digestion was done in 0.1 M sodium acetate buffer, pH 5.6, containing 0.001 M $CaCl_2$ using 0.1-1.0 unit of enzyme in a total digest volume of 200 μL; incubation was performed at 37 °C for the specified time. Influenza virus neuraminidase 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 $\mu$g 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 $\mu$Ci 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 $\mu$Ci 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.

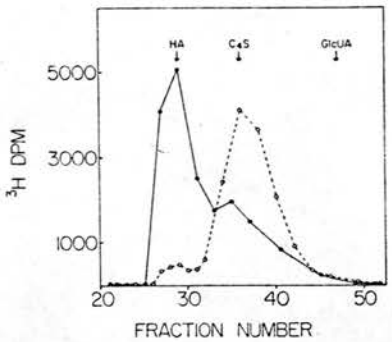



FIGURE 1: Fractionation of 0.2 (broken line) and 0.4 M (solid line) NaCl fractions on a 1.5 × 115 cm column of CPG 10-240 glass beads using 0.5 M $CaCl_2$ as the eluting solvent. Four-milliliter fractions were collected and 50-μL aliquots analyzed for radioactivity. The peak elution positions of marker 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-

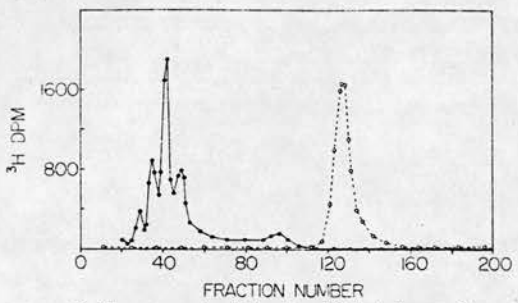



FIGURE 2: Chromatography of class I and II sialoglycopeptides on a DEAE-cellulose column (0.9 × 70 cm). Elution was with a linear gradient of 0.01 to 1.0 M pyridine acetate buffer, pH 5.1. Rate of flow was 30 mL/h. Fractions of 2.5 mL were collected and aliquots analyzed for radioactivity. Classes I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

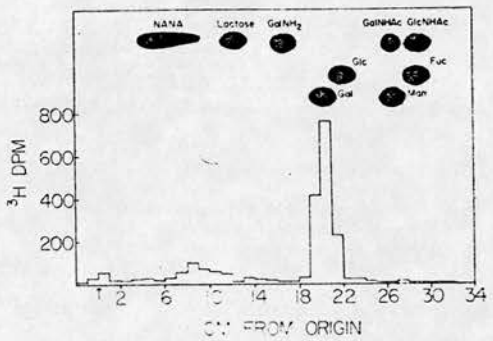



FIGURE 4: Paper chromatography of $^3$H-labeled neutral sugars isolated from class I sialoglycopeptides in solvent system B. Standard saccharides were visualized with a silver nitrate stain.

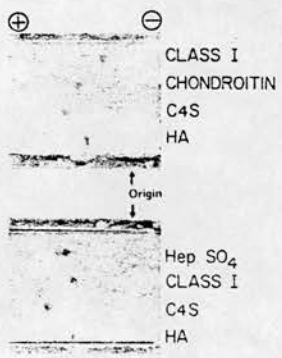



FIGURE 3: Cellulose acetate electrophoresis of class I sialoglycopeptide. *Upper*, in 0.2 M calcium acetate, 5 mA, 3 h; *lower*, in 0.1 N pyridine formate, pH 3.0; 10 mA, 20 min. Strips were stained with Alcian blue. Marker saccharides used were HA and C4S (see Figure 1), Hep-SO$_4$, heparan sulfate, and mouse melanoma chondroitin.

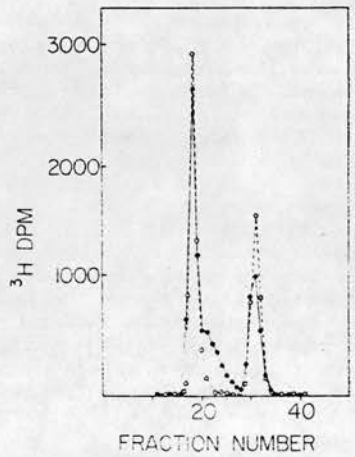



FIGURE 5: Gel filtration of class I sialoglycopeptide on a Bio-Gel P2 column (0.9 × 70 cm) after treatment with *Vibrio cholerae* neuraminidase (○) or treatment with neuraminidase followed by pronase (●). The low-molecule-weight material with peak position at fraction 31 had an elution profile identical 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 AG1 (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).

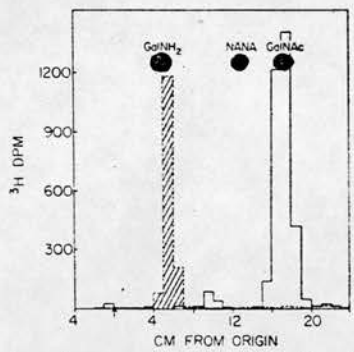



FIGURE 6: Paper chromatography of the low-molecular-weight material obtained by digesting asialo class I glycopeptide with *endo*-α-*N*-acetylgalactosaminidase before (shaded) and after (unshaded) treatment with Jack bean β-galactosidase. The chromatogram was developed in solvent system A and the standard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

*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 [$^3$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 AG1 (formate) columns. The sialylated oligosaccharides were eluted from the AG1 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*-

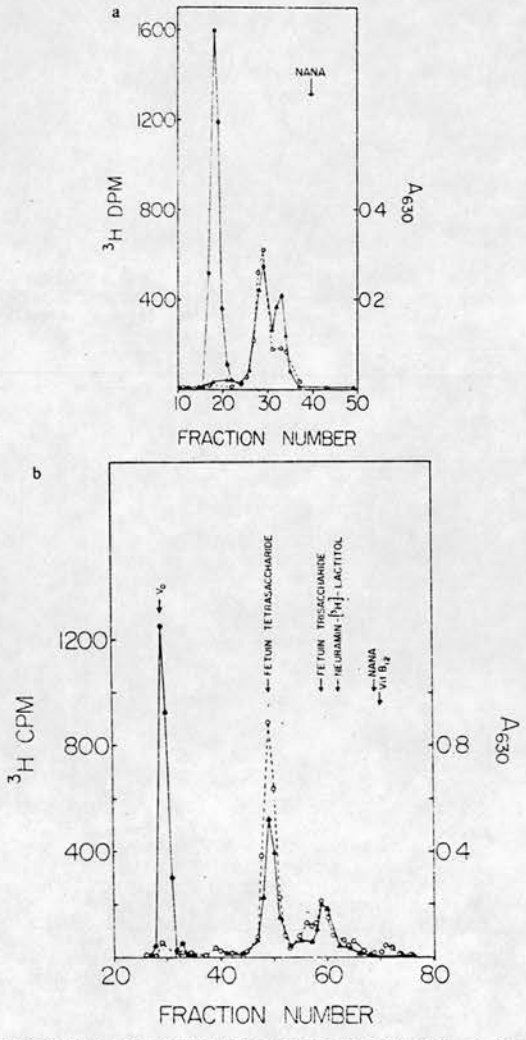



FIGURE 7: Gel filtration of class I sialoglycopeptides before (●) and after (○) treatment with alkaline borohydride on a (a, top) Bio-Gel P4 column (0.9 × 70 cm) and a (b, bottom) Bio-Gel P6 column (0.9 × 104 cm). The treated samples were mixed with tetrasaccharides and trisaccharides isolated from alkaline 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.

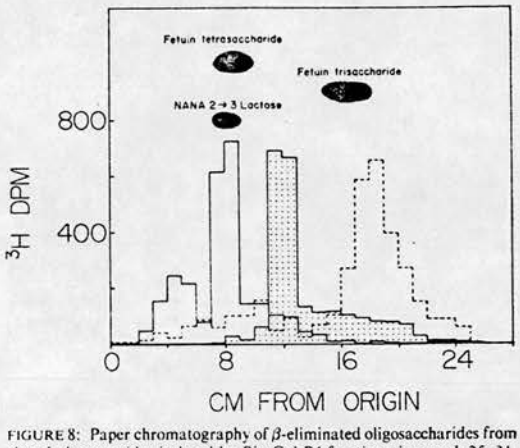



FIGURE 8: Paper chromatography of β-eliminated oligosaccharides from class I glycopeptides isolated by Bio-Gel P4 fractionation; peak 25–31, solid line; and peak 32–37, broken line. Chromatography was in solvent system A. The dotted area shows the mobility of neuramin[$^3$H]lactitol. Positions of other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

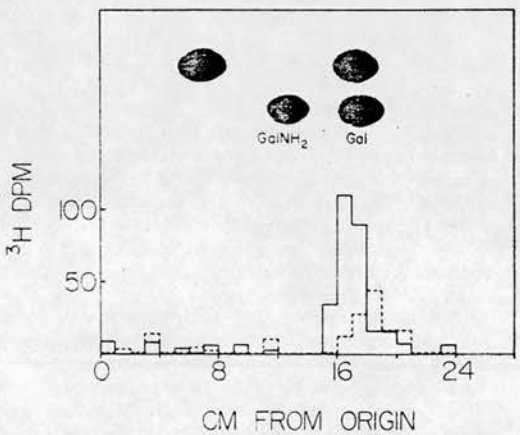



FIGURE 9: Paper chromatography of acid hydrolysates of sialyl oligosaccharides from class I glycopeptides before (broken line) and after (solid line) periodate oxidation. Chromatography was in solvent system D. The standards ($GalNH_2$ and Gal) and the products obtained by similar treatment of fetuin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

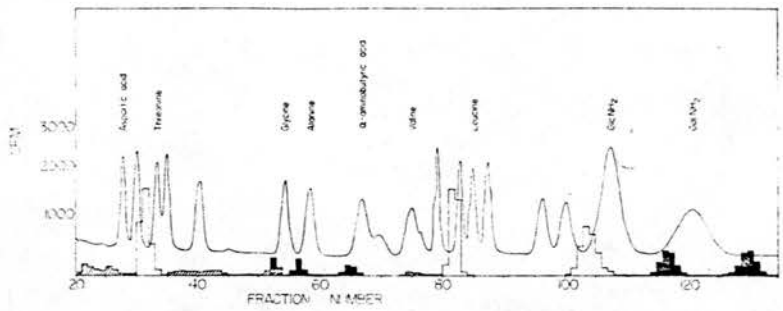



FIGURE 10: Analysis on the amino acid analyzer using a stream-splitting technique of the 6 N HCl hydrolysate of alkaline borotritide ($B^3H_4$) treated class I glycopeptides. The elution of tritium activity from the sample is shown by the black area and from the $NaB^3H_4$ control by the shaded area. 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 $\alpha$-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 $\beta$ 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

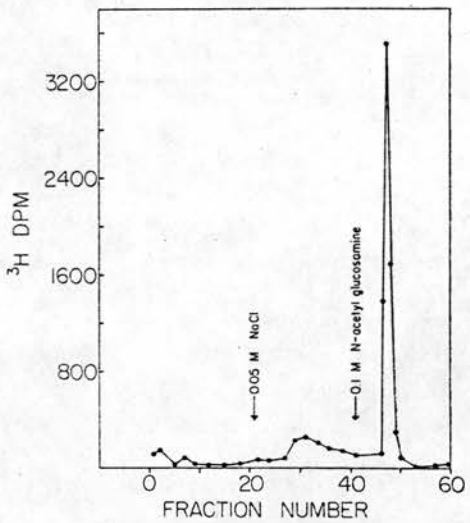



FIGURE 11: Affinity chromatography of class I sialoglycopeptides on a WGA Sepharose column. Elution was with 0.05 M Tris-HCl, pH 8.0, followed by 0.05 M NaCl in buffer, and finally *N*-acetylglucosamine. One milliliter fractions were collected and analyzed for radioactivity.

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-

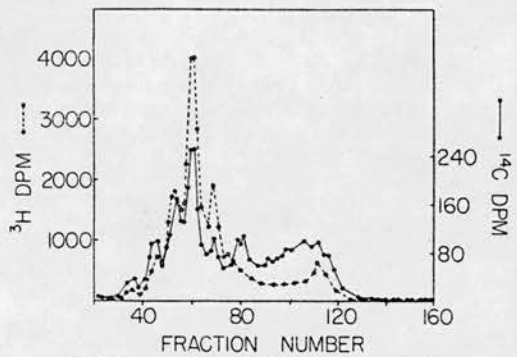



FIGURE 12: Cochromatography of $^{14}$C-labeled Tos-PheCH$_2$Cl-trypsinate components (●) and class II sialoglycopeptides (○) on a DEAE-cellulose column. Elution was with a linear gradient of 0.01 M pyridine acetate (300 mL) to 1.0 M pyridine acetate (300 mL). Fractions of 3.5 mL were collected and 1-mL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

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.

*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.

## 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 $\beta$-galactosidase and $\alpha$-*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($\beta$)$\rightarrow$[$^3H$]-Gal*N*Ac on digesting the asialo glycopeptide with *endo*-$\alpha$-*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 $\beta$-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 $\beta$-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 $\alpha$-[$^3H$]aminobutyric acid after $\beta$ 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 $\alpha$-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 $\beta$ 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-Phe$CH_2$Cl-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-Phe$CH_2$Cl-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-Phe$CH_2$Cl-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 $\beta$-galactosidase.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 252, No. 23, Issue of December 10, pp. 8609–8614, 1977
*Printed in U.S.A.*

# Purification and Properties of an Endo-α-*N*-acetyl-D-galactosaminidase from *Diplococcus pneumoniae**

(Received for publication, December 6, 1976)

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**An enzyme that hydrolyzes the *O*-glycosidic linkage between α-*N*-acetyl-D-galactosamine and serine or threonine in mucins and mucin-type glycoproteins was purified by chromatography on an Affi-Gel 202 column or isoelectric focusing from filtrates of *Diplococcus pneumoniae* cultures. The final preparations were free of protease and a wide range of other glycosidase activities. The preparation obtained by isoelectric focusing was shown to consist of a single protein by gel filtration and sodium dodecyl sulfate-gel electrophoresis. This preparation had an apparent molecular weight of about 160,000, determined by gel filtration, an optimum pH of 7.6, and an isoelectric point in the range pH 8 to 9.**

**The enzyme releases the disaccharide Gal-GalNAc from a variety of glycopeptide and glycoprotein substrates and appears to have a specific requirement for an unsubstituted galactose in the nonreducing terminus and an α linkage between *N*-acetylgalactosamine and the aglycone. This is the only endoenzyme known capable of cleaving the linkage between a carbohydrate and serine or threonine residues in glycoproteins.**

**The ability of this enzyme to act on macromolecular substrates and its pH optimum makes it ideally suited to explore the distribution and function of mucin-type glycoproteins on normal and cancer cell surfaces.**

During an investigation of the carbohydrate structure of sialoglycopeptides produced by mouse melanoma cells, we found that a partially purified neuraminidase from *Diplococcus pneumoniae* liberated the disaccharide galactosyl-*N*-acetylgalactosamine in addition to sialic acid. Preliminary results identifying the enzyme responsible for this as an endo-α-*N*-acetyl-D-galactosaminidase cleaving the *O*-glycosidic linkage between *N*-acetylgalactosamine and serine or threonine residues have been published (1). Endo and Kobata (2) have independently described this activity. An endogalactosaminidase has been described which cleaves GalN-GalN linkages in an oligogalactosaminoglycan but has no action on the *N*-acetylated oligogalactosaminoglycan (3).

* These studies were supported by Contract 1 HR 52954 and Grant CA 15483 from the United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The endoglycosidase described in this paper together with the endo-β-*N*-acetyl-D-glucosaminidases (4, 5) and the endo-β-D-galactosidases (6) isolated from various sources provide valuable tools for the investigation of the structure and function of glycoproteins.

In this paper we report the purification and characterization, including detailed studies on the substrate specificity, of an endo-α-*N*-acetyl-D-galactosaminidase isolated from culture filtrates of *D. pneumoniae*.

## EXPERIMENTAL PROCEDURES

*Materials* – Affi-Gel 202 and Bio-Gel P-2 (<400 mesh) were purchased from Bio-Rad Laboratories. Sephadex G-25 (Fine), G-200 (Fine), and marker proteins for molecular weight determination were purchased from Pharmacia. [1-$^{14}$C]Acetic anhydride (0.25 mCi/4.7 mg), [$^{3}$H]acetic anhydride (50 Ci/mmol), and Na$B^{3}H_4$ (204.8 mCi/mmol) were purchased from New England Nuclear.

*p*-Nitrophenyl derivatives of α-L-fucoside, α-D-mannoside, β-D-*N*-acetyl-D-glucosaminide, *O*-nitrophenyl-β-D-galactoside, and azocasein were purchased from Sigma Chemical Co. Phenyl-α-*N*-acetyl-D-galactosaminide was obtained from Nakarai Chemicals (Japan).

Neuramin-α2 → 3 lactose was prepared from skimmed bovine colostrum as described (7). Monosialoganglioside ($GM_1$) was provided by Dr. C.-L. Schengrund of this department and asialo ($GM_1$) was prepared by acid hydrolysis in 1 M HCOOH for 1 h at 100°. Porcine submaxillary mucin was generously provided by Dr. M. de Salegui, New York University Medical School. Antifreeze glycoprotein was kindly donated by Dr. R. E. Feeney, University of California at Davis. GalNAc α1 → 3(Fuc α1 → 2)Gal β1 → 4 GlcNAc β1 → 3 hexene-1,2,5,6-tetrol and Gal α1 → 3 (Fuc α1 → 2)Gal β1 → 4 GlcNAc β1 → 3 hexene-1,2,5,6-tetrol were kindly donated by Dr. E. A. Kabat, Columbia University. Mouse melanoma sialoglycopeptide (Class I) was isolated from [$^{3}$H]glucosamine-labeled cells as described (8, 9). Human melanoma sialoglycopeptides were isolated by procedures similar to those used for the mouse melanoma glycopeptides.[1] Human bronchial glycoprotein was isolated from bronchial secretions.[2] Fetuin and ovalbumin were purchased from Gibco and Sigma Chemical Co., respectively.

Asialoglycoproteins and glycopeptides were prepared by mild acid hydrolysis (0.1 N $H_2SO_4$, 80°, 1 h) or by neuraminidase digestion under the conditions described (8), followed by resolution of the products by exclusion chromatography on Sephadex G-25.

Ovalbumin asparaginyl oligosaccharide with the composition $(Man)_3(glcNAc)_2$-Asn was prepared according to the method of Huang *et al.* (10). This glycopeptide was labeled in the asparagine moiety by *N*-acetylation with [1-$^{14}$C]acetic anhydride as described by Koide and Muramatsu (4). 3-Hexene-1,2,5,6-tetroyl oligosaccharides were labeled by oxidation with galactose oxidase followed by

[1] V. P. Bhavanandan, J. R. Banks, and E. A. Davidson, unpublished results.

[2] P. A. Feldhoff, V. P. Bhavanandan, and E. A. Davidson, unpublished results.

reduction with $NaB^3H_4$ according to the procedure reported by Takasaki and Kobata (6). The $^3H$-labeled oligosaccharides were purified by chromatography on a column of Amberlite AG 50W-X8 ($H^+$) followed by gel filtration on a Bio-Gel P-2 column.

*Enzymes* – Partially purified preparations of *Diplococcus pneumoniae* glycosidases were generously provided by Dr. G. Ashwell, National Institutes of Health, Bethesda, Md. Neuraminidase (EC 3.2.1.18) from *Vibrio cholerae* and Pronase CB were purchased from Calbiochem; galactose oxidase (EC 1.1.3.9) was from Sigma Chemical Co.; horseradish peroxidase (EC 1.11.1.7) was from Worthington Biochemical and α-L-fucosidase (EC 3.2.1.51) was from Miles Laboratories.

*Analytical Methods* – Neutral sugar was determined by the anthrone reaction of Trevelyan and Harrison (11). The hexosamine and amino acid contents of the glycopeptides were assayed by amino acid analysis after hydrolysis with 4 N HCl at 100° for 6 h or in 6 N HCl at 110° *in vacuo* for 24 h, respectively. Sialic acid was assayed by a modification of Warren's thiobarbituric acid method (12) and bound sialic acid was determined after acid hydrolysis (0.1 N $H_2SO_4$, 80°, 1 h) by the same procedure.

Paper electrophoresis was performed at a potential of 39 V/cm for 2.5 h in 0.1 N pyridine acetic acid buffer, pH 5.4. The radioactivity on paper strips was detected using a Packard Radiochromatogram scanner, model 7201. When the activity was too low, particularly in the case of tritium-labeled materials, the position of the components on paper were determined by extraction and measurement as described (8).

*Preparation and Properties of Fetuin Glycopeptides* – Fetuin glycopeptides were prepared as described earlier (1) according to the method of Spiro and Bhoyroo (13). The glycopeptides in Fraction C were freed from a small amount of contaminating peptides by chromatography on Dowex 1-X2 (formate) and eluted with 0.1 N pyridine acetic acid buffer, pH 5.2. The yields of glycopeptide Fractions A, B, and C were 190 mg, 141 mg, and 49 mg, respectively, starting with 4 g of fetuin. The carbohydrate and amino acid compositions of these fractions were in general agreement with those published by Spiro and Bhoyroo (13).

Asialoglycopeptide Fraction C had the following composition; numbers in parentheses represent micromoles per 100 mg of asialoglycopeptide (galactose (134.8), *N*-acetylgalactosamine (115.2), threonine (32.4), serine (102.6), proline (118.8), glycine (59.4), alanine (84.0), and valine (19.2)). Glycopeptide Fraction A was composed mostly of *N*-glycosidically linked carbohydrates since 97% of the hexosamine was glucosamine; the sialic acid content of this fraction was 83.4 μmol/100 mg.

*Preparation and Properties of Porcine Submaxillary Mucin Glycopeptides* – The glycopeptides from porcine submaxillary mucin (0.5 g) having both blood group $A^+$ and $A^-$ active components were prepared by pronase digestion (14). The glycopeptide obtained after Sephadex G-25 chromatography was ultrafiltered through a UM-2 membrane (Amicon) and the retained glycopeptides further fractionated on Sephadex G-50 (1). The filtrate consisting of the smaller glycopeptides was separated into two fractions (PSM[3] UM-A and PSM UM-B) by gel filtration on Sephadex G-25 using 0.1 N pyridine acetic acid buffer, pH 5.2, as eluant. The higher (PSM UM-A; 32.4 mg) and lower (PSM UM-B; 21.4 mg) molecular weight fractions were found to have galactosamine/galactose ratio of 2.08:1 and 1.54:1, respectively. The former consists of $A^+$ PSM, glycopeptide and the latter is a mixture of both $A^+$ and $A^-$ glycopeptides.

Fucose and sialic acid residues from the PSM UM-A and PSM UM-B glycopeptides were removed by acid hydrolysis in 1 M HCOOH at 100° for 1 h and the galactosamine re-*N*-acetylated (15) to yield the asialo-afuco-PSM glycopeptides.

Enzymatic release of fucose or sialic acid from the PSM glycopeptides was achieved by incubation with α-L-fucosidase or neuraminidase at the optimum pH at 37° for 24 h. The glycopeptide was recovered by gel filtration on a column of Sephadex G-25.

*Preparation of $^3H$-labeled PSM ($A^+$) Glycopeptides (PSM UM-A)* – The sialic acid and/or fucose-free PSM ($A^+$) glycopeptides were labeled by treatment with galactose oxidase followed by $NaB^3H_4$ reduction according to the method of Morell *et al.* (16).

The reaction mixture, after labeling, was adjusted to pH 5, freed from borate by repeated evaporation with methanol and purified by gel filtration on Sephadex G-25 and, finally, by paper electrophoresis. The product gave a single radioactive band on paper chromatography and on paper electrophoresis. The product was hydrolyzed (4 N HCl, 100°, 6 h) and examined by paper chromatography. It was found that 90% of the tritium label was in galactosamine and the rest in galactose.

*Enzyme Assays* – The standard assay for endo-α-*N*-acetyl-D-galactosaminidase was performed in a reaction mixture composed of 1 μmol of Tris/maleate buffer, pH 7.6, 115 nmol (based on *N*-acetylgalactosamine) of asialofetuin glycopeptide Fraction C and an appropriate amount of enzyme in a total volume of 50 μl. After incubation at 37° for 3 h, 50 μl of water was added and the mixture was assayed for the released disaccharide (Gal 1 → 3 GalNAc) by the Morgan-Elson reaction (17) using one-fifth volume. Under these conditions, the reaction was linear with time (to 6 h) and enzyme amount. The identity of the released product as galactosyl-β-1 → 3-*N*-acetylgalactosamine and not free *N*-acetylgalactosamine was confirmed by gel filtration on a Bio-Gel P-2 column followed by paper chromatography (1). One unit of the enzymatic activity is defined as the amount of enzyme that hydrolyzes 1 μmol of substrate/min.

Exo-α-*N*-acetyl-D-galactosaminidase and neuraminidase were assayed as reported previously (1), except that in some experiments fetuin glycopeptide Fraction A was used as substrate for neuraminidase instead of neuramin-α-2 → 3 lactose. β-D-Galactosidase were assayed using *O*-nitrophenyl-β-D-galactosidase (18).

Endo-β-*N*-acetyl-D-glucosaminidase activity was determined by incubation with $^3H$-labeled ovalbumin glycopeptide followed by gel filtration on a Bio-Gel P-2 column to identify GlcNAc-Asn (4). Endo-β-D-galactosidase activity was determined using $^3H$-labeled hexene-1,2,5,6-tetroyl oligosaccharides. The released $^3H$-trisaccharides were identified by gel filtration on a Bio-Gel P-4 column (6).

β-*N*-Acetyl-D-glucosaminidase and other exoglycosidases were assayed by using the corresponding *p*-nitrophenylglycosides at the optimum pH as described (18). Proteases were assayed by incubating azocasein (1 mg) with the enzyme preparation in 220 μl of 0.1 M Tris/HCl buffer, pH 7.6, at 37° for 3 h. The reaction was terminated by addition of 780 μl of 8% trichloroacetic acid, and the absorbance of the supernatant measured at 350 nm. Appropriate controls with trypsin were also carried out.

*Isoelectric Focusing* – Analytical and preparative isoelectric focusing was done with an LKB apparatus using Ampholine carrier ampholytes according to the manufacturers instructions.

## RESULTS

### *Purification of Endo-α-N-acetyl-D-galactosaminidase was Achieved by Two Independent Methods*

*Method I* – Fig. 1 illustrates the purification of the endoenzyme on an Affi-Gel 202 column; the endoenzyme was eluted just before neuraminidase. The pooled fractions, after the addition of bovine serum albumin to 0.01% to prevent inacti-

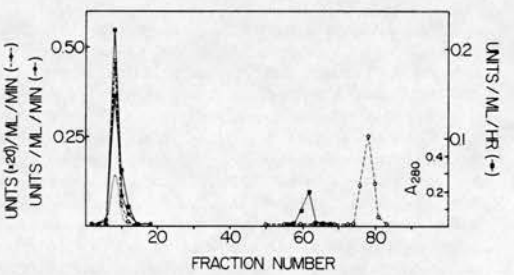


FIG. 1. Chromatography of the endo-α-*N*-acetyl-D-galactosaminidase on an Affi-Gel 202 column. A partially purified (18) mixture of *Diplococcus pneumoniae* glycosidases (300 μl) was applied to a column of Affi-Gel 202 (2.0 × 25 cm) equilibrated with 0.02 M Tris/HCl buffer, pH 7.5. The column was washed with 35 ml of the above buffer and then eluted by a concave NaCl gradient with a system composed of 45 ml each of the above buffer in the two mixing vessels and 45 ml of the same buffer containing 0.5 M NaCl in the reservoir. Fractions of 1.7 ml were collected. ■——■, endo-α-*N*-acetyl-D-galactosaminidase; ○- - -○, neuraminidase; ●——●, β-*N*-acetyl-D-glucosaminidase.

[3] The abbreviations used are: PSM, porcine submaxillary mucin; dansyl, 5-dimethylaminonapththalene-1-sulfonyl.

vation, were concentrated to about one-tenth their original volume using a PM10 membrane (Amicon). This preparation is stable for at least 6 months when stored at 4°.

*Method II* – Isoelectric focusing in polyacrylamide gels was carried out on the partially purified glycosidases using Ampholine of pH range 3.5 to 10. The results of a typical experiment are illustrated in Fig. 2. The endo-α-*N*-acetyl-D-galactosaminidase migrated to the high pH region and was well separated from other endo- and exoglycosidases present in the original mixture (Fig. 2*a*). The results of a preparative experiment are illustrated in Fig. 2*b*; the endoenzyme migrated to the pH region of 8 to 9 (see "Discussion"). The fractions having endoenzyme activity were pooled, the pH adjusted to 7.5 with 0.2 M $Na_2HPO_4$ and the solution concentrated to about one-tenth the original volume by dialysis against polyethylene glycol 20,000. The last traces of Ampholine were removed by gel filtration on a column of Sephadex G-25. The recovery of the endoenzyme activity was quantitative.

In a third method tested, chromatography of the mixture of glycosidases on a DEAE-Sephadex A-25 column was performed using a linear NaCl gradient as described by Endo and Kobata (2). The neuraminidase co-eluted with the endoenzyme and as such this method was not suitable for the separation of these two enzymes.

### *Purity of Enzyme*

The endo-α-*N*-acetyl-D-galactosaminidase preparations obtained by Methods I and II were free from neuraminidase, exo-α-*N*-acetyl-D-galactosaminidase, α-L-fucosidase, β-*N*-acetyl-D-glucosaminidase, β-D-galactosidase, α-D-mannosidase, endo-β-*N*-acetyl-D-glucosaminidase, and proteases. The preparation by Method II was also free of endo-β-D-galactosidase; the preparation by Method I was not assayed for this activity. Some preparations obtained by Method I, however, had trace amounts of neuraminidase and endo-β-*N*-acetyl-D-glucosaminidase. In separate experiments, endo-α-*N*-acetylgalactosaminidase was obtained completely free of neuraminidase from a preparation of culture filtrate of *D. pneumoniae* which had very low initial levels of neuraminidase and β-D-galactosidase.[4]

The purity of the endoenzyme was also examined by gel filtration on Sephadex G-200 and sodium dodecyl sulfate-polyacrylamide electrophoresis after labeling with [$^3$H]acetic anhydride. The endo-α-*N*-acetyl-D-galactosaminidase obtained by isoelectric focusing was concentrated about 140-fold by dialysis against polyethylene glycol 20,000 with retention of 70% of the enzyme activity. The enzyme solution was treated with [$^3$H]acetic anhydride (5 mCi) in 1 M sodium acetate at room temperature for 30 min. At this time, 10 μl of unlabeled acetic anhydride was added and the reaction was continued for 30 more min. The enzyme was freed from [$^3$H]acetic acid by dialysis against 0.05 M sodium phosphate buffer, pH 7.5, followed by gel filtration on a Sephadex G-25 column. The $^3$H-labeled preparation obtained was apparently denatured as it was devoid of endoenzyme activity.

Gel filtration of the [$^3$H]acetyl endoenzyme on Sephadex G-200 gave the results shown in Fig. 3. A single major peak was obtained which was located at nearly the same position as that of the endoenzyme activity. Two minor peaks are either degradation and aggregation products of the endoenzyme or other contaminating proteins.

[4] J. Umemoto, K. L. Matta, J. R. Banks, and V. P. Bhavanandan, unpublished results.

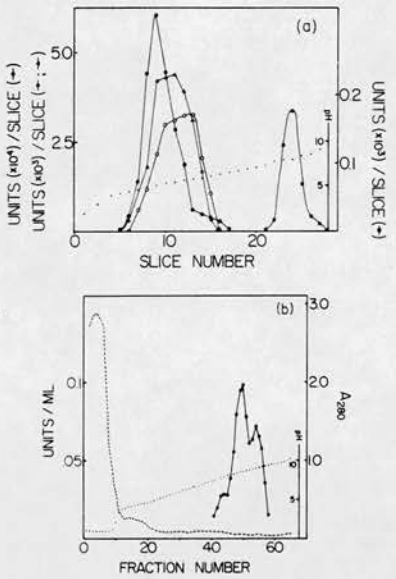


FIG. 2. Isoelectric focusing of the endo-*N*-acetyl-D-galactosaminidase (*a*) in a polyacrylamide gel and (*b*) in a column. (*a*) Twenty microliters of the enzyme preparation were applied to a 5-mm square of filter paper which was placed on the gel containing Ampholine of pH 3.5 to 10 and focused for 2.5 h at a constant power of 20 watts. At the end of the run, the gel was sliced longitudinally into 1-cm-wide strips and each strip further sectioned into 4-mm pieces. Pieces from a blank strip were suspended in 0.75 ml of distilled water and after overnight incubation at room temperature; the pH of the solutions were determined using a pH meter. Pieces from the sample strips were added to test tubes containing the appropriate enzyme assay mixtures and enzyme activity determined as described under "Experimental Procedures," except that Gal-GalNAc-α-*p*-nitrophenyl[4] was used as a substrate. ■——■, endo-α-*N*-acetyl-D-galactosaminidase; ▲——▲, β-D-galactosidase; ○——○, neuraminidase; ●——●, β-*N*-acetyl-D-glucosaminidase; · · · ·, pH. (*b*) Seven milliliters of the enzyme preparation were applied to a 110-ml column LKB 8108 containing 0.1% Ampholine of pH range 3.5 to 10 and focused at 300 V. The initial current of 5 mA dropped to 0.5 mA at the end of 50 h. At 72 h, the experiment was stopped and fractions of 2 ml were collected. After the pH was measured, enzyme activity was determined on suitable aliquots. ■——■, endo-α-*N*-acetyl-D-galactosaminidase; – – –, $A_{280}$; · · · ·, pH.

Polyacrylamide gel electrophoresis of the [$^3$H]acetyl endoenzyme on a 10% gel in the presence of 0.1% sodium dodecyl sulfate was done according to the method of Laemmli (19). At the end of the run, the gel was sliced into 2-mm wide segments and solubilized by incubation with 0.2 ml of 60% perchloric acid and 0.4 ml of 30% hydrogen peroxide at 60° for 4 h before measuring radioactivity. The results illustrated in Fig. 4 shows that enzyme migrated as a sharp band very close to a cytochrome *c* standard. Similar results were obtained on a 7% gel. The acetylated enzyme, when examined on a 6% gel at pH 4.3 but in the absence of sodium dodecyl sulfate, failed to enter the gel. The apparently low molecular weight of the acetylated enzyme on the sodium dodecyl sulfate gel suggests that the native material has several subunits, possibly as many as 8.

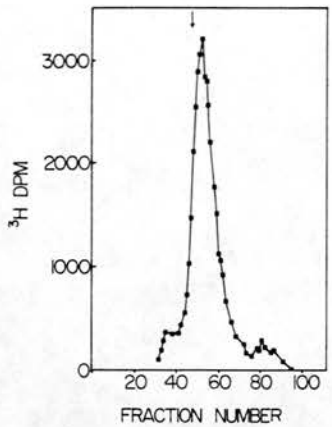


FIG. 3 (*left*). Sephadex G-200 filtration of [$^3$H]acetyl-endo-α-*N*-acetyl-D-galactosaminidase. $^3$H-labeled preparation (1.5 ml) containing $1.5 \times 10^5$ dpm was applied to a Sephadex G-200 column (2.6 × 95 cm). The column was eluted with 0.01 M Tris/HCl buffer, pH 7.5, containing 0.1 M KCl at a flow rate of 25 ml/h; fractions of 5.3 ml were collected. Aliquots were analyzed for radioactivity. The native endoenzyme was located by its enzyme activity, the peak position is indicated by an arrow.

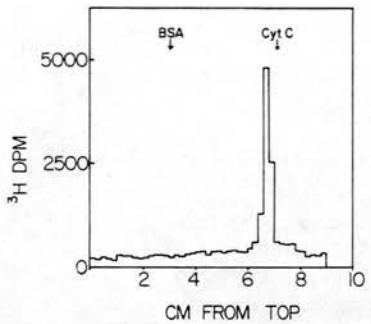


FIG. 4 (*center*). Radioactivity profile of [$^3$H]acetyl-endo-α-*N*-acetyl-D-galactosaminidase after sodium dodecyl sulfate polyacrylamide gel electrophoresis. Electrophoresis was performed in a 10% gel in the presence of 0.1% sodium dodecyl sulfate. The gel was sliced into 2-mm-wide segments. Each gel slice was solubilized and radioactivity was determined as described in the text. The marker proteins were located by Coomassie Blue G staining. *BSA*, bovine serum albumin; *Cyt. C*, cytochrome *c*.

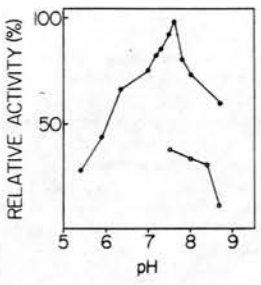


FIG. 5 (*right*). pH optimum of the endo-α-*N*-acetyl-D-galactosaminidase. The ezyme reaction was performed under standard conditions using 0.1 M Tris/maleate (●——●) or Tris/HCl (○——○) buffer of varying pH.

### *Properties of Endo-α-N-acetyl-D-galactosaminidase*

The activity of the enzyme with asialofetuin glycopeptide Fraction C as substrate at different pH in Tris/maleate buffer is shown in Fig. 5. The optimum pH is approximately 7.6; the same pH optimum was shown when 0.1 M sodium phosphate buffer, pH 5.6 to 8.1, was substituted for the 0.1 M Tris/maleate buffer. About 50% inhibition of activity was observed when Tris/HCl buffer was used, apparently due to chloride ions.

The molecular size of endo-α-*N*-acetyl-D-galactosaminidase, was estimated from its chromatographic mobility on a Sephadex G-200 column according to the method of Andrews (20). An apparent weight of 160,000 was obtained based on a series of standard proteins. The positions of the standards were determined by absorbance at 280 nm and that of the enzyme by its activity against asialofetuin glycopeptide Fraction C.

The effect of various cations and other reagents on the enzyme activity are summarized in Table I. Since inhibition by excess chloride ions was noted when Tris/HCl was used, the effect of cations on the enzyme was determined using acetate salts. The effect of galactose and sialic acid on the activity of the endoenzyme was measured by using asialofetuin glycopeptide Fraction C as substrate. The assay was performed under standard condition using 1.2 mM substrate and a 100 mM concentration of the monosaccharide. Sialic acid did not inhibit the enzyme but galactose gave a 70% inhibition.

The effect of varying substrate concentrations on the reaction rate with asialofetuin glycopeptide Fraction C was measured at 37° using 0.1 M Tris/maleate buffer, pH 7.6. The apparent Michaelis constant ($K_m$) was determined from the Lineweaver-Burk plot to be 1.0 mM.

The action of the endo-α-*N*-acetyl-D-galactosaminidase on saccharide, glycopeptide, glycoprotein, and glycolipid substrates is summarized in Table II. When $^3$H-labeled substances were used as substrate, the reaction products were detected by determination of the radioactivity after fractionation on Bio-Gel P-2 column, as described (1). In case of unlabeled substrates, the products were detected by color formation in the Morgan-Elson reaction (17). In both cases, the identity of the product was confirmed by paper chromatography.

TABLE I

*Effect of added substances on activity of endo-α-N-acetylgalactosaminidase*

Enzyme assay was performed under the standard condition in the presence of metal ion (acetate), EDTA, or cysteine for 6 h.

| | Concentration | Relative activity |
|---|---|---|
| | *mM* | % |
| None | | 100 |
| $K^+$ | 2 | 116 |
| $Na^+$ | 2 | 117 |
| $Ca^{2+}$ | 2 | 96 |
| $Mg^{2+}$ | 2 | 72 |
| $Mn^{2+}$ | 2 | 71 |
| $Hg^{2+}$ | 2 | 0 |
| EDTA | 2 | 60 |
| CySH | 2 | 88 |

## DISCUSSION

In our preliminary publication (1), we reported on the partial purification of the endo-α-*N*-acetyl-D-galactosaminidase on the Affi-Gel 202 column. The endoenzyme and neuraminidase eluted together from this column. Attempts to separate these enzymes by chromatography on Sepharosyl-glycyltyrosyl[N-(p-aminophenyl)]oxamic acid, an affinity ligand for neuraminidase (21), were not successful since the endoenzyme also bound to this column and eluted with the neuraminidase. Fractionation of the enzyme mixture on DEAE-Sephadex did

TABLE II

*Substrate specificity of endo-α-N-acetyl-D-galactosaminidase*

| Substrates | Structure | Hydrolyzed |
|---|---|---|
| Asialofetuin glycopeptide Fraction C<br>Asialo Afuco PSM($A^-$) glycopeptide | Gal1$\xrightarrow{\beta}$3GalNAc$\xrightarrow{\alpha}$Ser(Thr)<br>\| | + |
| Antifreeze glycoprotein* | Gal1$\xrightarrow{\beta}$3GalNAc$\xrightarrow{\alpha}$Thr→Protein | + |
| Afuco PSM($A^-$) glycopeptide | Gal1$\xrightarrow{\beta}$3GalNAc$\xrightarrow{\alpha}$Ser(Thr)<br>6<br>α\|<br>2<br>NeuNGc | - |
| B16 Mouse melanoma glycopeptide Class I | Gal$\xrightarrow{\beta}$[$^3$H]GalNAc$\xrightarrow{\alpha}$Ser(Thr)<br>\|<br>[$^3$H]NeuAc | - |
| Fetuin glycopeptide Fraction B<br>(mixture of tetra- and trisaccharides) | Gal1$\xrightarrow{\beta}$3GalNAc$\xrightarrow{\alpha}$Ser(Thr)<br>3 6<br>α\| α\|<br>2 2<br>NeuAc NeuAc | - |
| Asialo Afuco PSM($A^+$) glycopeptide | [$^3$H]GalNAcl$\xrightarrow{\alpha}$3Gall$\xrightarrow{\beta}$3GalNAc$\xrightarrow{\alpha}$Ser(Thr) | - |
| Asialo PSM($A^+$) glycopeptide | [$^3$H]GalNAcl$\xrightarrow{\alpha}$3Gall$\xrightarrow{\beta}$3GalNAc$\xrightarrow{\alpha}$Ser(Thr)<br>2<br>α\|<br>1<br>Fuc | - |
| Asialo mouse melanoma glycopeptide | Gal1$\xrightarrow{\beta}$3[$^3$H]GalNAc$\xrightarrow{\alpha}$Ser(Thr) | + |
| Asialo human melanoma glycopeptide | ——— | + |
| Asialo human bronchial glycoprotein† | ——— | + |
| Asialo PSM | ——— | + |
| Asialo Fetuin | ——— | + |
| Asialo $GM_1$ ganglioside | Gal1$\xrightarrow{\beta}$3GalNAcl$\xrightarrow{\beta}$4Gall$\xrightarrow{\beta}$4Glcl→1Ceramide | - |
| Ovine submaxillary mucin | NeuAc 2$\xrightarrow{\alpha}$6 GalNAc(Ser/Thr)Protein | - |

* Extent of hydrolysis was 30% after 24 hr incubation as determined by analysis of the galactose and galactosamine of the product.

† Seven percent of the galactosamine in the macromolecule was released as the disaccharide Gal→GalNAc after 24 hr incubation.

not succeed in separating the neuraminidase and endoenzyme activities. Purification of the endoenzyme was achieved by the two methods discussed under "Results."

In order to assess the absence of other proteins in the endoenzyme, preparations obtained by Affi-Gel 202 were dansylated (22), treated with fluorescamine (23), or [$^3$H]acetylated prior to electrophoresis on polyacrylamide gels in presence or absence of sodium dodecyl sulfate. These attempts were generally not successful in demonstrating the homogeneity of the protein due to insufficient material and because of denaturation during dansylation or aggregation of the apparently hydrophobic enzyme protein. The isoelectric focusing method yielded the endoenzyme in excellent yield and high purity. Using this preparation, we were successful in labeling the protein with [$^3$H]acetic anhydride without concomitant insolubilization. Gel filtration on Sephadex G-200 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of this preparation demonstrated the absence of other major extraneous proteins in our preparations. The slight shift in the peak positions of the enzyme activity and the [$^3$H]acetyl-labeled protein may be due to conformational effects arising as a result of the derivatization of basic residues. Sodium dodecyl sulfate-gel electrophoresis indicated that the endoenzyme may be composed of several subunits of relatively low molecular weight. The isoelectric focusing data illustrated in Fig. 2*b* show at least two peaks of activity. In some preparations of the endoenzyme, in addition to these peaks at the unusually high isoelectric point, a peak with an apparent pI of 5.0 was also detected. The enzyme with the lower pI seems to have a greater molecular weight than the high pI form. The exact relationship between the two forms of the enzyme is not clear and is under investigation. It is possible that the lower pI form is an association product formed during the isolation procedure. The enzyme has a rather high optimum

pH, (7.6), relative to that of other glycosidases, whether Tris/maleate, Tris/HCl, or phosphate buffers were used. Endo and Kobata (2) report a pH optimum of 6.0 in phosphate buffer for their enzyme preparation; the reason for this discrepancy is not clear but may be a function of the substrate employed and the time of incubation.

The hydrolysis of asialofetuin glycopeptide Fraction C by the purified endoenzyme was linear for more than 6 h. The extent of hydrolysis after 6 h was 45% and after 24 h, it was 94.3%. When mouse melanoma asialoglycopeptide Class I was used as substrate, the enzyme released 75% of the disaccharide in 24 h. This substrate has Gal → GalNAc linked to serine and threonine apparently in clusters (8, 9) similar to that found in glycophorin (24). This suggests that the enzyme is capable of hydrolyzing the *O*-glycosidic linkage between Gal → GalNAc and serine or threonine even when they occur sequentially on the polypeptide chain.

Antifreeze glycoprotein (25) was also a good substrate for this enzyme. In this glycoprotein, the disaccharide, Gal → GalNAc, is linked entirely to threonine. These results together with the fact that almost 95% of Gal → GalNAc linked to serine and threonine in asialofetuin glycopeptide Fraction C was released suggests that the enzyme can act on both serine and threonine linkages. Preliminary results indicate that the enzyme can also efficiently hydrolyze the synthetic substrate Gal → GalNAc-α-*p*-nitrophenyl.[4] Thus it is possible that the nature of the aglycone is not critical for the activity of the enzyme.

The inability of the enzyme to hydrolyze substrates such as NeuAc → Gal → GalNAc-Ser/Thr, GalNAc → Gal → GalNAc → Ser/Thr, GalNAc → (Fuc) → GalNAc-Ser/Thr, NeuAc → GalNAc-Ser/Thr together with the fact that galactose is an inhibitor of the enzyme suggests that a nonreducing galactose terminus is necessary for the recognition of the substrate by the enzyme. It is possible that galactose is involved in the binding of the substrate to the active site. Whether the enzyme has strict specificity for a GalNAc residue in the penultimate position is not clear from the present studies. The glycopeptide Gal → (NeuNGc) → GalNAc → Ser/Thr was not hydrolyzed by the enzyme; therefore, it seems that the GalNAc has to be unsubstituted, at least in position 6. The enzyme failed to release Gal → GalNAc from asialo ($GM_1$) ganglioside suggesting that the β-*N*-acetylgalactosaminyl linkage is not recognized by the enzyme.

The availability of endo-β-*N*-acetyl-D-glucosaminidases (4, 5) which cleave the chitobiose unit of the saccharide core of serum-type glycoproteins has facilitated the removal of the bulk of carbohydrate units linked *N*-glycosidically to protein. A most useful property of the endoenzyme described in this paper is its ability to hydrolyze macromolecular substrates. The enzyme released the disaccharide Gal → GalNAc from mouse melanoma asialoglycopeptide Class I ($M_r$ = 10,000 to 12,000), antifreeze glycoprotein, asialofetuin, asialo-PSM, and asialo human bronchial mucin ($M_r$ = about $2 \times 10^6$).[2] This should be of utility in both structural and biosynthetic studies. In addition, the ability of the endo-α-*N*-acetyl-D-galactosaminidase to act on macromolecular substrates coupled with a pH optimum close to physiological should be extremely valuable in investigating the distribution of *O*-glycosidically linked oligosaccharides on cell surfaces.

*Acknowledgments*—We are most grateful to Dr. Gilbert Ashwell for providing generous quantities of the partially purified enzyme preparation used in these studies. We also thank Dr. M. de Salegui for pig submaxillary mucin, Dr. R. E. Feeney for antifreeze glycoprotein, Dr. E. A. Kabat for oligosaccharides, and Perry Habecker and John Banks for technical assistance.

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[CANCER RESEARCH 37, 4336-4345, December 1977]

# Chemical and Biological Properties of B16 Murine Melanoma Cells Grown in Defined Medium Containing Bovine Serum Albumin[1]

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## SUMMARY

The addition of 1% (w/v) bovine serum albumin (BSA) to the F12 medium utilized for the growth of the B16 melanoma cells significantly stimulated the growth of this cell line. The synthesis of mucopolysaccharides and sialoglycopeptides in this medium is identical with that in Eagle's minimal essential medium with Earle's balanced salt solution supplemented with 2 mM L-glutamine, twice the recommended concentration of vitamins, nonessential amino acids, sodium pyruvate, and 10% (v/v) fetal calf serum. Cell volume and morphology did not change significantly under the different growth conditions and tumorigenicity, as assayed by injection of cultured cells into syngeneic animals, was not decreased. Analysis of the BSA used indicated the presence of a sialoglycoprotein contaminant. This sialoglycoprotein contaminant was present in all lots examined and contains *N*-acetyl- and *N*-glycolylneuraminic acid, mannose, galactose, and glucosamine. The sialoglycoprotein can be removed by chromatography on acetate form anion-exchange resin at pH 4.3. F12 media containing the purified BSA plus selenite and the sodium salts of palmitic, oleic, and linoleic acids supported growth of the melanoma cells to the same extent as did the media containing unpurified BSA, indicating that the sialoglycoprotein has no role in sustaining the growth of the cells.

## INTRODUCTION

Previous reports from this laboratory have described the anionic saccharides (sialoglycopeptides and mucopolysaccharides) made by human (2) and mouse (19) melanoma cells, SV40- and herpes simplex virus-transformed hamster cells (20), and suitable controls. These studies and those by other groups involved the characterization of radioisotopically labeled molecules that had been synthesized by cells during their growth *in vitro* and *in vivo* from radiolabeled precursors such as glucosamine, fucose, sulfate, glucose, and acetate (9, 12, 23). The prior investigations were often of a comparative nature and were subject to uncertainties due to differences in the dilution or pool size of a labeled precursor, entrapment by serum components (1), carbohydrate concentration, and other variations in the culture media. Thus, it is highly desirable to confirm incorporation data by direct mass analysis. This has been done in several instances (8, 9), but not in all.

The cell surface anionic saccharides of the mouse melanoma B16 are reduced in amounts under growth conditions that result in the loss of tumorigenicity (21). These same conditions result in an increased expression of cell surface antigens (24). The mouse melanoma is useful for immunological studies, since it is tumorigenic in syngeneic animals (21), since it can be cultured *in vitro* while retaining tumor-forming ability, and since X-irradiated cells inoculated into syngeneic mice are capable of protecting the animals against challenge by viable melanoma cells (6). Therefore, it seems reasonable that syngeneic mice can develop an immune response to these cells and that the antigenic components are associated with the plasma membrane (18). Soluble components synthesized by these cells have been studied in an attempt to identify possible tumor-specific antigens (7).

Prior studies on the B16 mouse melanoma with the use of labeled precursors resulted in the isolation of 2 complex saccharide components that are either not produced or are produced in much reduced quantities by a control population of mouse iris melanocytes. The 2 components have been characterized as a high molecular weight chondroitin sulfate (19) and a mucin-type glycoprotein (4, 19). For examination of their immunological importance, it is necessary to isolate reasonable quantities of these components. Since our data also showed that large amounts of these components are present in a soluble form in conditioned culture medium, the possibility of isolating them from this source seemed attractive. This would be feasible, provided these cells could be adapted to grow in a chemically defined medium without the loss of their biological and biochemical properties. In this instance, the properties would be anionic polysaccharide synthesis, ability to proliferate, and tumorigenicity. The studies reported in this paper deal with the effect of medium composition on these properties of an amelanotic variant of the B16 mouse melanoma.

## MATERIALS AND METHODS

**Cells.** The amelanotic variant of the B16 mouse melanoma cell line described previously was used in these

[1] Supported in part by USPHS Grant CA15483 and Grant 1 P30 CA18450-01, awarded by the National Cancer Institute, Department of Health, Education, and Welfare.

[2] To whom requests for reprints should be addressed.

Received February 10, 1977; accepted August 30, 1977.

experiments (19). The phenotype was not stable, and these cells produce a small amount of melanin which can be detected by visual observation.

**Media.** Cells were routinely maintained in a modified version of MEM[3] (11) containing 10% FCS (Flow Laboratories, Inc., Rockville, Md.), 0.1 mM concentrations of each nonessential amino acid, 1 mM sodium pyruvate, 2 mM L-glutamine, twice the recommended concentration of vitamins, and penicillin (10 units/ml) and streptomycin (10 $\mu$g/ml); this is termed MB16 medium with FCS. The medium was obtained from Flow Laboratories as a dried powder. The nonessential 100× amino acid solution, 100× sodium pyruvate solution, 100× MEM vitamin solution and 100× L-glutamine solution were obtained from Grand Island Biological Co. (Grand Island, N. Y.) and added to the liquid medium prepared from the powder.

F12 medium as described by Ham (13) was used for growth experiments. This was obtained as a dry powder from Flow Laboratories. This medium was supplemented in all cases by addition of 2 mM L-glutamine, 0.11% $NaHCO_3$, penicillin (10 units/ml) and streptomycin (10 $\mu$g/ml) after filtration through a 0.45-$\mu$m Nalgene filter unit (Fisher Scientific Co., Pittsburgh, Pa.). The albumin was added to the F12 medium and filtered immediately after dissolving. Routine cultures indicated that the medium was not contaminated with bacteria, yeast, mold, or mycoplasmas (19).

**Reagents.** Tritiated glucosamine (D-glucosamine [6-$^3$H]hydrochloride, 7.3 Ci/mmole) and $Na_2{}^{35}SO_4$ (carrier-free; specific activity, 800 mCi/mmole) were obtained from New England Nuclear, Boston, Mass. D-Glucosamine [1-$^{14}$C]-hydrochloride (specific activity, 51.5 mCi/mmole) was also obtained from New England Nuclear.

BSA, Cohn Fraction V, was obtained from Sigma Chemical Co., St. Louis, Mo. Lots 55C-0074, 95C-0191, 86C-0140, and 126C-0199 were used for the experiments; these preparations contain $\alpha$-globulins as the principal contaminant. Pentex BSA Cohn Fraction V, 99% pure, presumably prepared by the classical Cohn low-temperature alcohol precipitation method, was obtained from Miles Laboratories (Catalog No. 81-003, Lot 234). The defatted BSA was obtained from Sigma (Catalog No. A-6003, Lot 46C-7450). Crystalline BSA was obtained from ICN, La Puente, Calif. (Lot 18008, Catalog No. 1136). Sodium oleate (Catalog No. 0251), sodium linoleate (Catalog No. L5378), and sodium palmitate (Catalog No. P2010) were all approximately 99% pure and were obtained from Sigma. Selenous acid (Catalog No. A-286) was obtained from Fisher Scientific Co.

**Growth Promotion Protocol.** In order to test growth-promoting ability, we seeded the cells into 16-oz glass bottles (Brockway Glass Co. Inc., Brockway, Pa.) at $2 \times 10^6$ viable cells in 25 ml of medium. Smaller-scale experiments were initiated by seeding $7 \times 10^5$ viable cells in 4-oz glass bottles (Brockway) in a total of 10 ml of medium. After the allowance of 12 to 24 hr in the serum-free medium for the cells to attach, the culture medium was changed to that to be tested for growth-promoting ability. The cells grown in the MB16 were fed every 2 days, while those in F12 medium were fed daily, since the low concentrations of some components in this medium might become exhausted and limit growth.

When not used for growth studies, the cells were passed at biweekly intervals and were harvested using phosphate-buffered saline-0.02% ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*'-tetraacetic acid (3). Cell counts were obtained with the use of a hemacytometer, and the viability of the cells was determined by trypan blue exclusion.

**Tumorigenicity Test Protocol.** Each of 10 C57BL/6J mice was inoculated s.c. with 0, $10^4$, $10^5$, or $10^6$ sixth-passage melanoma cells grown in F12-1% BSA medium; a control group received cells grown in MB16 containing 10% heat-treated FCS. The cells were injected s.c. in 0.2 ml of BSA-free F12 or serum-free MB16, respectively.

**Cell Sizing Protocol.** The cells used for this study were all in the first passage and were grown under identical conditions except for the medium. The cells were resuspended in Isoton (Scientific Products, Edison, N. J.) at $8 \times 10^4$ cells/ml and sized with a calibrated Model B Coulter counter with frequent agitation of the cell suspension.

**Labeling of Complex Saccharides and Their Extraction.** Cells in the logarithmic growth phase were fed with medium containing [$^{14}$C]glucosamine (0.6 $\mu$Ci/ml). Growth was allowed to proceed for 48 hr in 16-oz glass bottles or in a 690-sq cm roller bottle (Bellco Glass, Inc., Vineland, N. J.). The complex saccharides were isolated by previously described methods (19).

**Complex Saccharide Analysis.** After precipitation of the $^{14}$C-labeled material with CPC, the nonprecipitable and the precipitated materials were analyzed as described previously (4, 19). Further characterization of the nonprecipitated fraction was performed after dialysis by chromatography on 0.9- x 70-cm DE-52 (DEAE-cellulose; Whatman Inc., Clifton, N. J.) column with the use of a 0.01 to 1 M pyridine acetate, pH 5.2 gradient. The precipitated sialoglycopeptide that was eluted by 0.4 M NaCl from the precipitated material was characterized by its ability to bind to a wheat germ agglutinin:Sepharose 4B column and its elution from that column with 0.1 M *N*-acetylglucosamine (4).

**Assays.** Sialic acid determinations were performed by the thiobarbituric acid method (28) or the periodate: resorcinol procedure (14). *N*-Acetylneuraminic acid obtained from Pfanstiehl or Difco was used as standard. *N*-Glycolylneuraminic acid was isolated from porcine submaxillary mucin.

Paper chromatography of sialic acids was carried out with *n*-butyl acetate:glacial acetic acid:$H_2O$ (3:2:1) (25), and the sugars were detected by the spray method of Warren (29).

Paper chromatography of the neutral sugar fraction was carried out after a 4-hr hydrolysis of the sample in 1 N HCl (26) and passage through a mixed-bed resin, Dowex 50 ($H^+$):Dowex 1 (formate). The eluate which contained >90% of the neutral sugar present before hydrolysis was chromatographed in *n*-butyl alcohol:pyridine:$H_2O$ (6:4:3), and the sugar present was detected by a $AgNO_3$ spray (27).

Hexosamine content was determined after 4 hr at 100° hydrolysis in 4 N HCl with the use of the Elson-Morgan

[3] The abbreviations used are: MEM, Eagle's minimal essential media with Earle's balanced salt solution; FCS, 56° 30-min heat-treated fetal calf serum; MB16, Eagle's MEM as above supplemented with 2 mM L-glutamine, twice the recommended concentration of MEM vitamins, nonessential amino acids, and sodium pyruvate; BSA, bovine serum albumin; CPC, cetyl pyridinium chloride; CPG, controlled-pore glass beads.

reaction as modified by Boas (5) after Dowex 50 ($H^+$) chromatography of the hydrolysate and 2 N HCl elution.

Protein concentration was measured according to the method of Lowry *et al.* (15) with the use of BSA as the standard.

Gas-liquid chromatography of the saccharides as the trimethylsilyl derivatives of methylglycosides was done as described previously (4). The appropriate corrections for detector response and losses during hydrolysis were applied.

Chondroitinase ACII and *Vibrio cholerae* neuraminidase susceptibility assays were done by molecular size profiles (19). After a 24-hr incubation in a volume of 500 $\mu$l, the sample was lyophilized and applied in $H_2O$ to a previously calibrated CPG 10-240 80 to 120 mesh glass bead column; individual fractions were assayed for radioactivity. Changes in molecular weight to a region coincident with authentic chondroitin sulfate disaccharide or sialic acid indicated degradation of the species under test.

**Chromatographic Purification of BSA.** Chromatography of the Sigma BSA (Lots 95C-0191, 86C-0140, and 126C-0199) was performed on AG 1-X2 200 to 400 mesh resin (Bio-Rad Laboratories, Richmond, Calif.) converted to the acetate form. A 4- x 40-cm column was equilibrated with 1 mM sodium acetate buffer, pH 4.3. The sample (1 g) was dissolved in water, dialyzed for 24 hr *versus* 6 liters of deionized $H_2O$ at 4° followed by 6 liters of 1 mM sodium acetate buffer, and loaded onto the column. The column was eluted with a linear gradient consisting of 900 ml each of 1 and 50 mM sodium acetate buffer, pH 4.3 (Chart 1). Fractions 115 to 205 and 206 to 240 were combined, dialyzed, and lyophilized to yield 840 and 115 mg, respectively. The sialic acid contents of these fractions together with the starting material are given in Table 4. The sialic acid contaminant present in the BSA was cleanly separated from the albumin with the use of this method, although the trailing edge of the peak contained a small amount of periodate:resorcinol-positive material. The main portion of the albumin peak was used for the growth studies. This material was shown to have a molecular weight of 66,000 by gel exclusion chromatography and was readily soluble in deionized water after dialysis and lyophilization.

**Photography and Scanning Electron Micrographs.** Photographs were taken with the use of a Zeiss inverted microscope with phase-contrast optics equipped with a 35-mm semiautomatic camera system.

For scanning electron micrographs, cells were fixed with glutaraldehyde:formaldehyde (by the method of Karnovsky) for 20 to 30 min at 4°. They were rinsed with 0.1 M cacodylate buffer, dehydrated in a graded ethanol series, and then dried at the critical point, with the use of liquid $CO_2$, and coated with 200 Å of gold and palladium by ion sputtering.

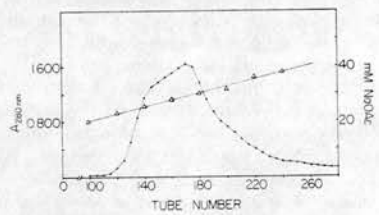


Chart 1. Purification of BSA (Sigma) on an AG 1-X2 200 to 400 mesh column (4 x 40 cm). The column was eluted with a linear gradient of 1 to 50 mM sodium acetate (*NaoAc*) (△). The fractions were assayed for protein by measuring absorbance at 280 nm (●).

## RESULTS

Growth data representative of 4 separate experiments for the various media are summarized in Table 1. The results demonstrate that the cells survived in both F12 and MB16 media over the course of the experiment (84 hr) with little, if any, loss of viability. The growth rate is the same in the F12 or MB16 when the medium is supplemented with 10% FCS. The cells grow slightly better in 0.5% BSA-F12 medium than they do in MB16 supplemented with 1% FCS. However, F12 supplemented with 1% BSA (F12-1% BSA) provides significantly better growth than does the MB16-1% FCS medium.

A typical titration curve for cells grown in F12 medium plus varying BSA concentrations indicates that optimal growth is obtained at 1% BSA, and no further effect is noted by increasing the BSA concentration to 2% (Chart 2). The growth rate is only slightly slower than that for cells grown in 10% FCS. The plating efficiency was >50% (typically 70%) in this medium, and the growth of single cells into colonies could be seen with inoculations of 500 cells. Osmolality determination indicated that the F12 medium and F12 medium supplemented with 1 or 2% BSA or 10% FCS had an osmolality of 286 ± 3 mOsmoles/kg, while the MB16 medium with 10% FCS had an osmolality of 272 ± 3 mOsmoles/kg.

The cell line could be grown for multiple passages in the F12-1% BSA medium while showing high viability, high density, melanin production (Fig. 1), and a fairly reproducible growth rate. In a typical experiment, the cells were

Table 1

*Growth data for B16 mouse melanoma cells in serum-containing and serum-free media*

The cells were seeded into 16-oz glass bottles at 2 × $10^6$ viable cells/25 ml medium. After 12 hr, the time of first medium change, the number of cells was 80 to 100% of that seeded. The apparent doubling times were calculated on the assumption that growth started at 12 hr postseeding. The cell numbers listed are those at 84 hr postseeding. Data represent an average of 2 separate experiments.

| Media | Cell no. × $10^{-6}$ | Apparent doubling time (hr) |
|---|---|---|
| MB16 | 2.2 (87)[a] | |
| MB16 + 0.1% FCS | 2.2 (87) | |
| MB16 + 1% FCS | 5.0 (87) | 54 ± 4 (2)[b] |
| MB16 + 10% FCS | 21 (97) | 22 ± 2 (2) |
| F12 | 2.1 (87) | |
| F12 + 0.5% BSA | 7.3 (95) | 44 ± 4 (2) |
| F12 + 1% BSA | 11 (95) | 36 ± 7 (2) |
| F12 + 2% BSA | 11 (97) | 36 ± 7 (2) |
| F12 + 10% FCS | 21 (98) | 22 ± 2 (2) |

[a] Numbers in parentheses, percentage of viability.
[b] Numbers in parentheses, number of samples analyzed.

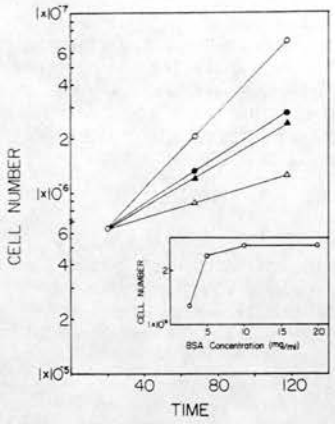


Chart 2. Growth of B16 melanoma cells in F12 medium containing 0.25 (△), 0.5% (▲), 1 or 2% (●) BSA or 10% FCS (○). *Inset*, growth with respect to albumin concentration. The results presented are averages of duplicates.

grown for 6 passages in 16-oz bottles and seeded at 2 × $10^6$ cells/bottle (viable) at each passage. Under these conditions, when passaged at 7-day intervals, cell densities of 2 to 2.5 × $10^7$ viable cells were obtained. The cells could also be grown under these conditions in 690-sq cm roller bottles when seeded at 2 × $10^7$ cells; over a 7-day period, the cell number increased to $10^8$ cells at 95% viability. The cells were frozen in 10% dimethyl sulfoxide after the second passage in F12-1% BSA and could be revived after 1 year in a liquid $N_2$ freezer.

After 6 passages the cells grown in F12-1% BSA were tested for tumor-forming ability as compared to the same cells grown in MB16 medium containing 10% FCS; the results (Table 2) indicate no loss of tumorigenic activity. There was no significant change in cell volume in the various media tested (B16-10% FCS, F12-1% BSA, F12-10% FCS; data not illustrated).

**Effect of Reduced FCS Concentration or Desialylation of the FCS on Complex Saccharide Synthesis.** Cells were grown to confluency in B16 media-10% FCS and subsequently labeled for 48 hr with [$^3$H]glucosamine and $Na_2{}^{35}SO_4$ in the presence of B16 10, 1, or 0.1% FCS. The incorporation of radioactivity into macromolecules was determined as described under "Materials and Methods"; the results are shown in Table 3. A decrease in the rate of synthesis of saccharides extractable into 0.4 or 2 M NaCl with decreasing FCS concentration can be seen. Analysis of the 0.4 M NaCl eluate by affinity chromatography on a wheat germ agglutinin:Sepharose column and eluting with 0.1 N *N*-acetylglucosamine indicated the presence of the Class I sialoglycopeptide (4) in cells labeled in the presence of reduced amounts of FCS (1 or 0.1%) (Chart 3).

Since one of our aims was to isolate mg quantities of sialoglycoprotein produced by these cells, the possibility of growing the cells in desialyzed serum-containing media was also examined. Desialyzed FCS was prepared by acid (0.1 N $H_2SO_4$, 80°, 1 hr) treatment and extensive dialysis. Analysis by periodate:resorcinol and thiobarbituric acid methods showed that between 95 and 99% of the sialic

Table 2

*Tumor-forming ability of B16 mouse melanoma cells grown in serum-containing and serum-free media*

Cells were prepared and injected s.c. aseptically in 0.2 ml of the serum-free or BSA-free medium. The C57BL/6J mice were observed for 2 months; data are presented as number of tumors present/number of animals given injections.

| | Growth media | |
|---|---|---|
| Dose | B16-10% FCS | F12-1% BSA |
| $10^6$ | 9/10 | 10/10 |
| $10^5$ | 7/10 | 10/10 |
| $10^4$ | 3/10 | 1/7 |

acid was removed. The growth of cells in medium containing 10% desialyzed FCS showed that the cells could not attach in this medium but that the medium was capable of promoting growth if the cells were first seeded in regular medium and then switched to the test medium.

Cells were grown to confluency in B16 medium-10% FCS and then labeled for 48 hr with [$^3$H]glucosamine and $Na_2{}^{35}SO_4$ in the presence of the medium or B16 medium-10% desialyzed FCS. The results showed that there was no quantitative difference in the incorporation of $^{35}$S into nondialyzable macromolecules, whether the cells were labeled in the presence of FCS or desialyzed FCS. However, the $^3$H label incorporated into macromolecules was reduced by about 30% when the cells were labeled in the presence of desialyzed FCS.

**Effect of Growth in F12-1% BSA on Complex Saccharide Synthesis.** Cells grown in F12-1% BSA (Lots 55C-0074 and 95C-0191) were labeled by the use of [$^{14}$C]glucosamine, and the sialoglycopeptides were isolated from the spent medium as described under "Materials and Methods." The sialoglycopeptides in the 0.4 M NaCl fraction and in the CPC supernatant were examined for potential Class I and II sialoglycopeptides on CPG, wheat germ agglutinin: Sepharose 4B, and DEAE-cellulose columns. The elution pattern of the 0.4 M fraction on the CPG column is similar to that of the Class I (mucin-type) sialoglycopeptides produced by these cells, which elutes at the position of chondroitin sulfate (13,000 daltons) (4). After treatment with *V. cholerae* neuraminidase, about 50% of the radioactivity moved in the low-molecular-weight region, and the balance eluted in the original position. The amino sugar analysis of this fraction indicated that 75% was galactosamine and the balance was glucosamine. The presence of the Class I sialoglycopeptide in this fraction was confirmed by chromatography on a wheat germ agglutinin:Sepharose 4B column. The radioactivity that was bound specifically (61%) was recovered by elution with 0.1 M *N*-acetylglucosamine and dialysis; on rechromatography, this fraction bound completely to the lectin column.

The CPC supernatant fraction was mixed with $^3$H-labeled Class II sialoglycopeptides isolated from cells grown in regular (10% FCS) media and chromatographed on a DEAE-cellulose column with the use of a gradient of 0.01 to 1.0 M pyridine acetate, pH 5.2 (Chart 4). The coelution of the $^3$H- and $^{14}$C-labeled components illustrates the identity of the Class II sialoglycopeptides synthesized by cells grown in serum or in BSA-containing medium. The ratios of the $^3$H

Table 3

*Effect of FCS concentration on production of complex saccharides: 0.4 M NaCl eluate, Class I sialoglycopeptides, and 2 M NaCl eluate mucopolysaccharides*

Cells were grown to confluency from 2 × $10^6$ cells (initial inoculum) in 16-oz bottles and labeled with [$^3$H]glucosamine (5 μCi/ml) and $Na_2$ $^{35}SO_4$ (25 μCi/ml) for 48 hr. The media fractions were then prepared (19) and analyzed. The final cell counts of 1.1 × $10^7$ cells/bottle indicated that the cells were confluent at the start of the labeling period.

| | $^3$H | | | $^{35}$S | | |
|---|---|---|---|---|---|---|
| % concentration of FCS | dpm | %T[a] | dpm/$10^3$ cells | dpm | %T[a] | dpm/$10^3$ cells |
| | | | 0.4 M NaCl eluate | | | |
| 10 | 1,198,148 | 100 | 53 | 24,438 | 100 | 1.1 |
| 1 | 978,324 | 82 | 46 | 12,929 | 53 | 0.6 |
| 0.1 | 730,751 | 61 | 33 | 10,595 | 43 | 0.5 |
| | | | 2 M NaCl eluate | | | |
| 10 | 2,588,517 | 100 | 114 | 772,872 | 100 | 34 |
| 1 | 1,708,050 | 66 | 81 | 647,007 | 84 | 31 |
| 0.1 | 1,128,838 | 44 | 52 | 380,695 | 49 | 18 |

[a] %T, percentage of incorporation of the given serum concentration relative to that eluate isolated from cells grown in 10% FCS-containing media.

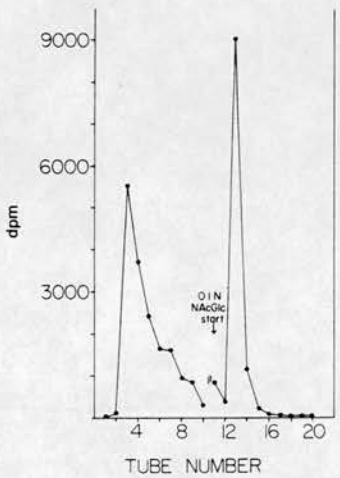


Chart 3. Chromatography of Class I sialoglycopeptides produced by melanoma cells grown in MB16-0.1% or 1% FCS medium on a wheat germ agglutinin:Sepharose 4B column. Elution was with 0.05 M Tris-HCl buffer, pH 7.8, followed by 0.1 M *N*-acetylglucosamine (*NAcGlc*) in the same buffer.

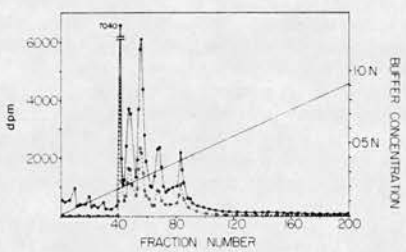


Chart 4. Chromatography of non-CPC-precipitable (Class II) sialoglycopeptides grown in the presence of [$^{14}$C]glucosamine in F12-1% BSA medium (○) and corresponding fractions from cells grown in the presence of [$^3$H]-glucosamine in MB16-10% FCS medium (●). The samples were mixed, applied on a DEAE-cellulose column, and eluted with a linear gradient of 0.01 to 1.0 M pyridine acetate, pH 5.2. The fractions were analyzed for $^3$H and $^{14}$C activity by scintillation counting.

dpm/peak to total recovered $^3$H dpm divided by $^{14}$C dpm/peak to total recovered $^{14}$C dpm were, from left to right: Peak 1, 0.72; Peak 2, 0.98; Peak 3, 1.06; Peak 4, 1.3; and Peak 5, 0.92. These ratios indicate both qualitative and quantitative similarities.

Analyses of the 2 N NaCl fraction indicated the presence of a high-molecular-weight material eluting in the position of the chondroitin 4-sulfate previously described (19). This fraction was completely susceptible to chondroitinase ACII. The low-molecular-weight components could not be completely characterized, due to the small amount of material present but appear to be heparan sulfate.

**Analysis of BSA for Growth-promoting Factor(s).** The results reported above are interesting in that the various lots of BSA promoted growth; it was not known whether the BSA itself or a contaminant was responsible for supporting growth. Reports in the literature suggest that the bound fatty acids of albumin (17) are responsible for the growth of various cell lines (13, 31). Because a major reason for the use of this medium is the isolation of cell-specified sialoglycopeptides and since commercial preparations of BSA are only ~96% pure, an analysis of the various BSA samples was undertaken. The periodate:resorcinol assay (14) showed the presence of significant amounts of sialic acid (Table 4), which was not dialyzable. For further identification of the sialic acid-containing contaminant, BSA (20 g) was extensively digested with pronase, and the nondialyzable glycopeptide fraction was recovered in the supernatant after 5% trichloroacetic acid precipitation at 4°. The sialic acid content of this glycopeptide mixture was 16%, as determined by the thiobarbituric acid assay after acid hydrolysis (28) and on the intact glycopeptide by the periodate:resorcinol method (14). The absorption spectra of the chromogens obtained by both

Table 4
*Sialic acid content of BSA preparations from various sources*

| BSA source | Sialic acid (mg/g) | |
|---|---|---|
| ICN crystalline | 2 | (1)[a] |
| Pentex 99% pure | 1.8–2 | (1) |
| Sigma Fraction V | 1.2–1.4 | (5) |
| Sigma Fraction Va[b] | 0 | (4) |
| Sigma Fraction Vb[b] | 0.05 | (2) |

[a] Numbers in parentheses, number of samples analyzed.
[b] These samples were prepared by chromatography of Sigma Fraction V on an AG 1-X2 column (Chart 1). Va and Vb are Fractions 115 to 205 and 206 to 240, respectively.

procedures were identical with those given by standard *N*-acetylneuraminic acid, thus excluding the possibility of spurious color formation due to the presence of deoxy sugars, lipid peroxidation, etc. (28). Further, sialic acid-free or fatty acid-free (Sigma) BSA at 10 mg/ml did not suppress or enhance chromogen formation when mixed with standard sialic acid (3 to 6 $\mu$g). The sialic acid present in the BSA preparations was isolated by acid hydrolysis (0.1 N $H_2SO_4$, 80°, 1 hr) followed by chromatography on an AG 1-X8 (formate) column and elution with 0.3 N formic acid. Paper chromatography (26) showed the presence of both *N*-acetyl- and *N*-glycolylneuraminic acids.

The neutral sugar content of the glycopeptide fraction was determined by the phenol sulfuric acid method (10) and was found to be 15% with the use of mannose as the standard. Galactose and mannose were the neutral sugars identified by paper chromatography. Hydrolysis with 6 N HCl in a vacuum, at 100° for 24 hr followed by analysis on the amino acid analyzer showed the presence of glucosamine as the only amino sugar; the hexosamine content was estimated to be 13% after correction for losses during hydrolysis. Gas-liquid chromatographic analysis of the glycopeptides indicated the presence of galactose, mannose, glucosamine, and sialic acid in the ratio of 3.2, 4.7, 2.7, and 2, respectively; the protein content was 22%.

The effect of the removal of this sialoglycoprotein from BSA on the growth of B16 cells was determined with the use of BSA purified by ion-exchange chromatography (Chart 1). F12 medium, when supplemented with 1% sialoglycoprotein-free BSA, $10^{-8}$ M sodium selenite, sodium palmitate (10 to 15 $\mu$g/ml), sodium oleate (10 to 15 $\mu$g/ml) and sodium linoleate (5 to 7.5 $\mu$g/ml) gave identical growth to F12 media supplemented with 1% untreated BSA of the same lot (Chart 5); sialoglycopeptide-free BSA without additional fatty acids also supported growth, but to a lesser extent. In these experiments, the cells cultured in F12 medium for 4 days immediately prior to the experiment (during which the cells had a doubling time of 68 hr) were used. This was done to minimize carry-over of growth-promoting activity. This cell line also had been growing in F12-1% BSA medium for 5 passages prior to growth in F12 medium. Supplementation of F12 medium with 1% defatted BSA (Sigma) and the additional ingredients noted previously gave identical results (Chart 6). Dialyzing and lyophilizing of the BSA had no effect on growth-promoting activity. Titrations indicated that selenite at $10^{-8}$ M in F12 medium is able to stimulate cell growth 27% over that of F12 medium alone; therefore, in the above experiments

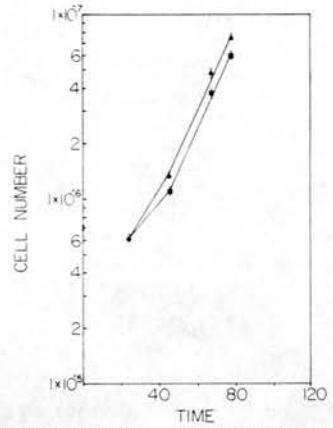


Chart 5. Growth of B16 melanoma cells in F12 medium containing 1% untreated BSA (▲); 1% sialoglycopeptide-free BSA supplemented with $10^{-8}$ M selenite and the sodium salts of the fatty acids (▲) and 1% sialoglycopeptide-free BSA plus $10^{-8}$ M selenite (■).

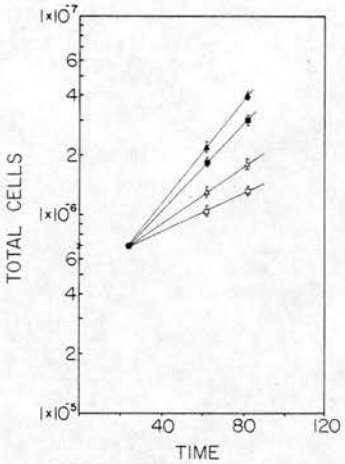


Chart 6. Growth of B16 melanoma cells in F12 medium supplemented with fatty acid-free BSA (Sigma) ± $10^{-8}$ M selenite (■), in F12 medium supplemented with fatty acids, fatty acid-free BSA (Sigma), and $10^{-8}$ M selenite or control (1% untreated BSA) (▲), in F12 medium only (□), and in F12 medium plus $10^{-8}$ M selenite (△). The cells used in this experiment were not adapted to growth in F12 medium.

selenite was added at this concentration to ensure optimal growth; however, it had no effect when added to medium containing unpurified albumin.

**Isolation of Sialoglycopeptides Produced by Mouse Melanoma Cells Grown in Chemically Defined Media.** Pooled F12-1% BSA-spent medium, about 2.5 liters, was dialyzed extensively, lyophilized, and digested with pronase, and the nondialyzable portion was isolated after dialysis and lyophilization (350 mg). This material gave the following analysis: sialic acid, 4.1%; hexosamine, 3.5%; neutral sugars, 6.0%; and uronic acid, 1.3%. The balance of the pronase-treated material (337 mg) was dissolved in

0.04 M $Na_2SO_4$, and CPC was added to 0.2% final concentration. After incubation for 16 hr at room temperature, the precipitate was collected by centrifugation and washed 3 times with 0.2% CPC in 0.04 M $Na_2SO_4$ by resuspension and centrifugation. The precipitate was then fractionally extracted with NaCl, dialyzed to remove CPC and NaCl, and lyophilized. The fractions corresponding to Class I sialoglycopeptides and mucopolysaccharides were 3.0 and 2.9 mg, respectively.

The results presented clearly demonstrate the ability of the mouse melanoma line B16 to make the Class I and II sialoglycopeptides (4, 19) and the anionic polysaccharide chondroitin sulfate (19), which is the high-molecular-weight component, when grown in F12 medium supplemented by specific lots of commerical BSA.

The biological properties of this line also appear unchanged compared to the cells grown in medium containing 10% FCS. During the first passage, the cells appear to grow somewhat more slowly than when serum is present in the culture medium, but the growth rate is still quite satisfactory. The cells grow to the same density as those grown in the FCS-containing media, can be grown in roller bottles, and do not become contact inhibited. Tumor-forming ability is not altered by the growth in this medium, and scanning electron microscope pictures show significant surface activity (30) (Fig. 2*a*). The size of the cells is not visibly altered by growth in serum-free medium, nor is their volume changed.

**Electron Microscopy.** Scanning electron micrographs of this cell line grown in F12-1% BSA medium or MB16-10% FCS indicated a similar amount of surface activity (Fig. 2).

## DISCUSSION

With respect to the parameters of lack of contact inhibition, tumor-forming ability, morphology, and volume, the cells grown in the BSA-containing medium are not detectably different from cells grown in the presence of serum. These results, coupled with the fact that the cells retain the ability to produce melanin and to make characteristic complex saccharides, indicate that this albumin-containing F12 medium can be substituted for that containing serum.

The Sigma lots of BSA (A4503) all stimulated growth, whereas more highly purified samples (Pentex, Inc., Kankakee, Ill.) did not stimulate growth to the same extent, nor did fatty acid-free BSA. The factor or factors present in the BSA that permit growth appear to be partially removed by chromatography on AG 1-X2 200 to 400 mesh anion-exchange resin under conditions that result in essentially quantitative recovery (95%) of the BSA from the column. The growth factor may not be the sialic acid-containing contaminant, as supplementation of F12 medium with the purified sialoglycoprotein fraction did not stimulate growth. Pentex BSA prepared by similar methods (Cohn Fraction V low-temperature alcohol precipitation) does not have the growth-promoting activity of the lots of Sigma BSA tested, despite the fact that it has greater amounts of sialoglycoprotein contamination. Fatty acid-free BSA (Sigma) also has poor growth-promoting activity, despite containing 80 to 100% of the sialoglycoprotein present in the nondefatted BSA, but retains optimal growth-promoting activity if supplemented with fatty acids and selenite. Chromatography under the conditions described may remove fatty acids (22), and selenite and different lots of BSA may vary in fatty acid content (22).

Despite the presence of the sialoglycoprotein contaminant, the unpurified BSA at the concentration used still contains 17 to 24 times less sialic acid than does the medium containing 10% FCS. The use of this medium containing 1% BSA, or preferably the fatty acid-supplemented medium with purified BSA, will facilitate the isolation, chemical, and immunological characterization of the complex saccharides elaborated by these cells in culture. Although this is only an intermediate step to the desired growth of cells in protein and macromolecule-free medium, it should facilitate the above objectives. It is possible to grow the B16 melanoma cells in F12 medium, but the growth was not of the same magnitude or consistency as with F12 medium supplemented with BSA. This might be due to the presence of selenite (16) in trace amounts, since not all lots of the F12 media supported growth to the same extent. The capability of the cells to grow better in the presence of serum albumin may be due to the capability of this protein to solubilize and transport fatty acids (17) and possibly cofactors such as biotin.

A recent communication (J. Mather and G. Sato, Hormone Requirements of a Clonal Line of the B16 Mouse Melanoma, personal communication) has described the growth of another mouse melanoma line in a serum-free (Dulbecco's modified MEM) medium. This medium, however, contains insulin (5 $\mu$g/ml), testosterone ($10^{-9}$ M), luteinizing hormone-releasing factor (10 ng/ml), transferrin (5 $\mu$g/ml), and follicle-stimulating hormone (0.4 $\mu$g/ml); the last 2 are glycoproteins. In this medium, the synthesis of the normal mucopolysaccharides and (sialo)glycopeptides has not been examined.

It is of interest that wheat germ agglutinin cytotoxicity is increased 6-fold in F12-1% BSA medium over that in 10% FCS-containing medium for these cells (L. Evans and E. A. Davidson, unpublished observation).

## ACKNOWLEDGMENTS

We are thankful to Dr. C.-L. Schengrund who generously provided a Zeiss inverted microscope with phase-contrast optics equipped with a 35-mm semiautomatic camera system and to Mordecai Moore for help with the amino sugar analysis.

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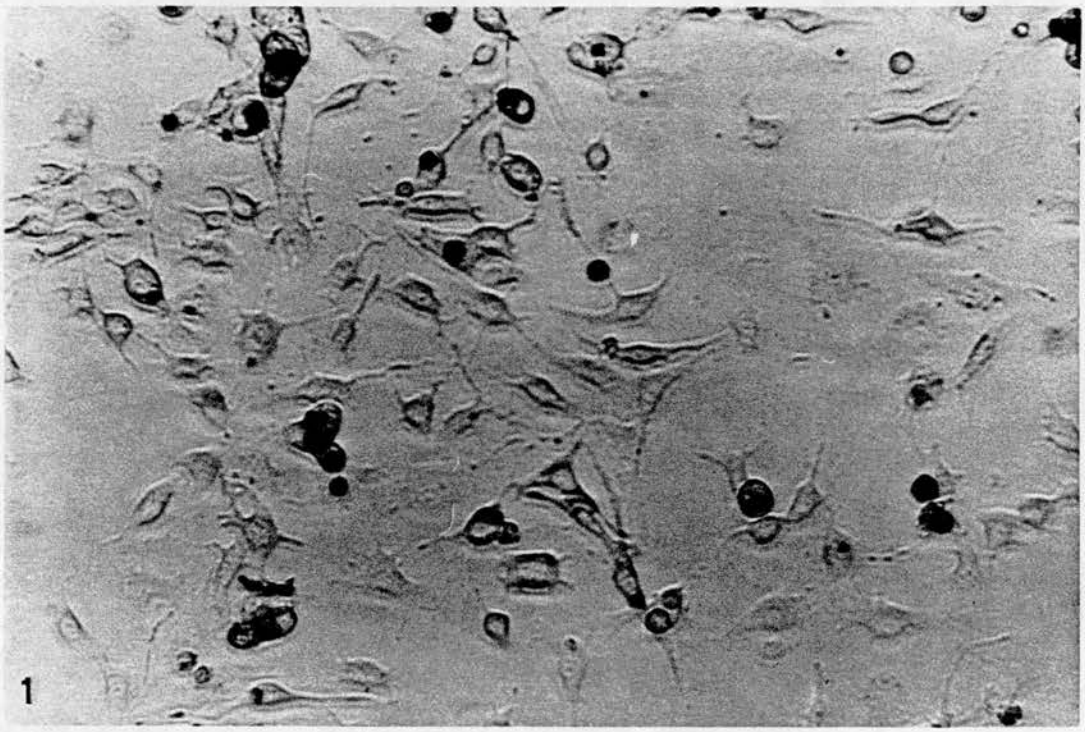


Fig. 1. Photomicrograph of cells grown in F12-1% BSA. The dark granules are melanin granules. × 666.

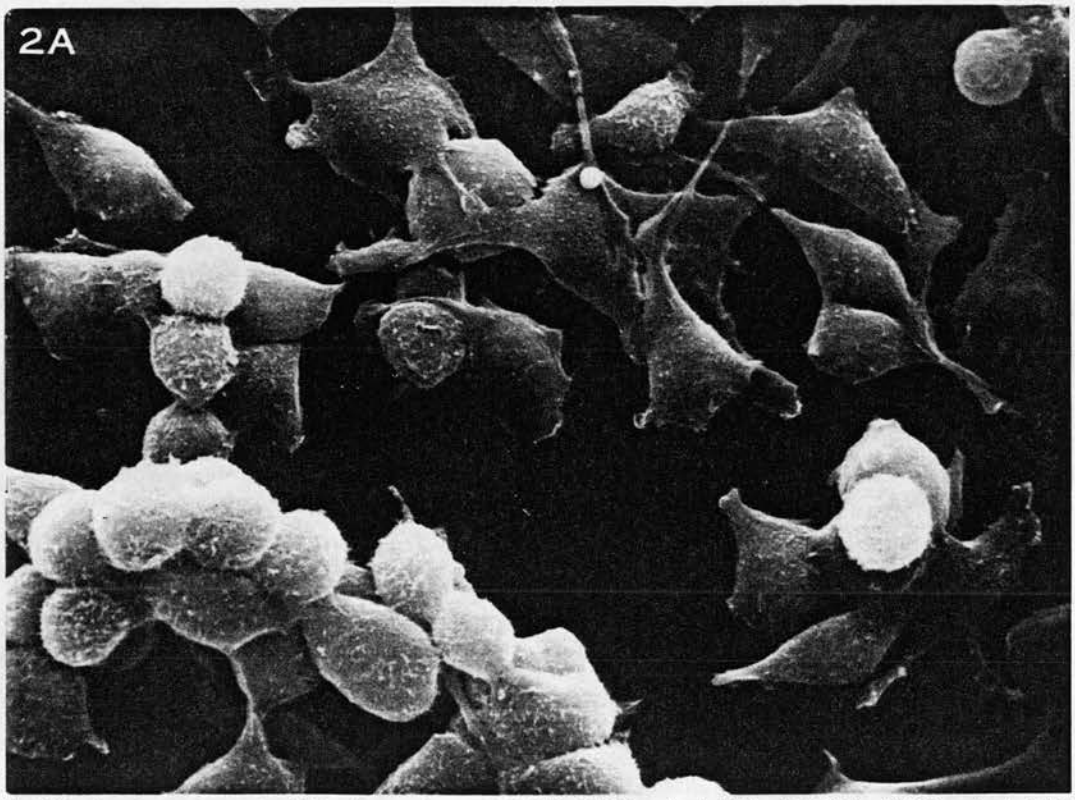


Fig. 2. Scanning electron micrographs of B16 melanoma cells grown in (*A*) F12 + 1% BSA medium (× 2250) and (*B*) MB16 + 10% FCS medium (× 2270).

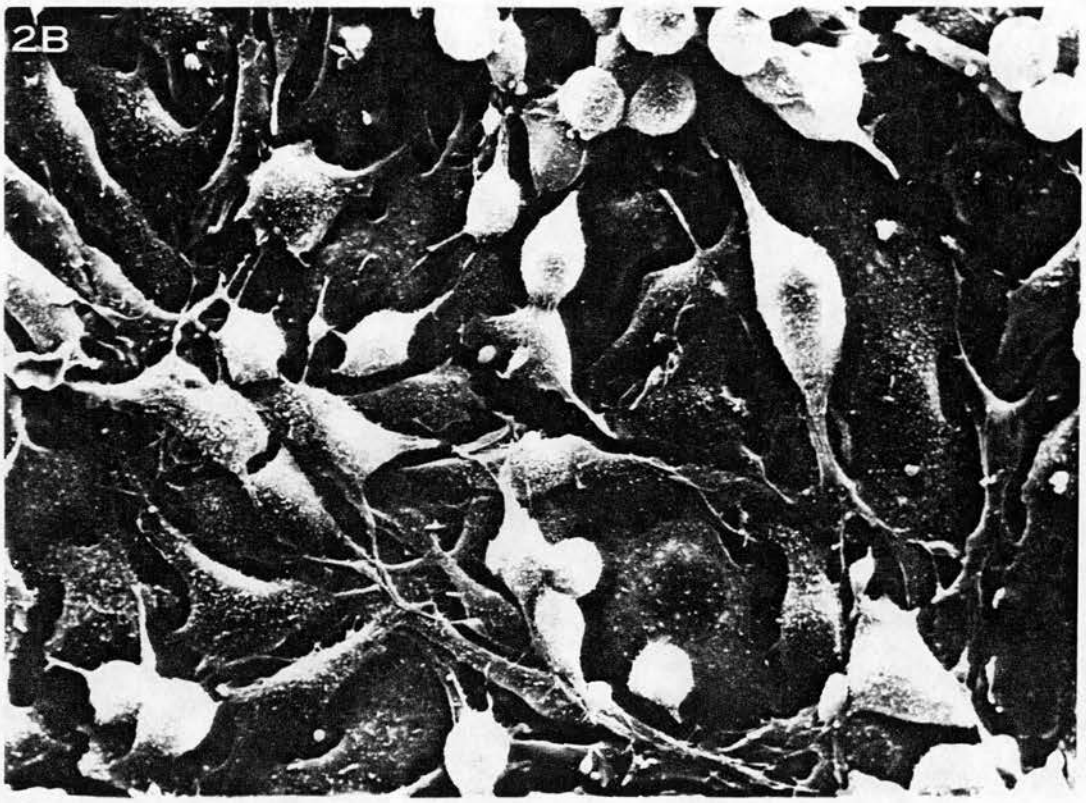


Fig. 2*B*

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*Carbohydrate Research*, 65 (1978) 73–83


# ISOLATION AND CHARACTERIZATION OF GLYCOCONJUGATES FROM B16 MOUSE MELANOMA TUMORS

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(Received June 9th, 1977, accepted for publication in revised form, September 1st, 1977)

## 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 $\beta$-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.

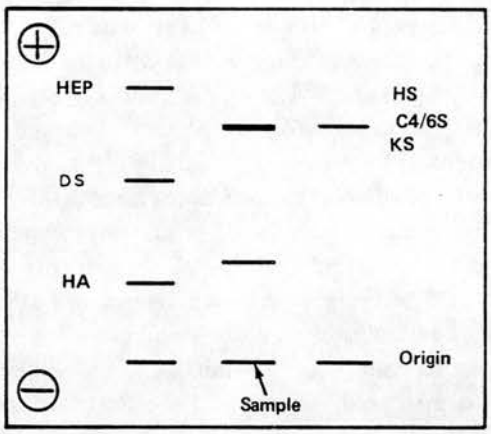


Fig. 1. Cellulose acetate electrophoresis of the 2.0M NaCl Fraction in 50mM LiCl–10mM HCl, pH 2.0 at 1 mA per cm for 20 min. The components were detected with the Alcian Blue stain. Abbreviations: Hep, heparin; HS, heparan sulfate; C4S, chondroitin 4-sulfate; C6S, chondroitin 6-sulfate; KS, keratan sulfate;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

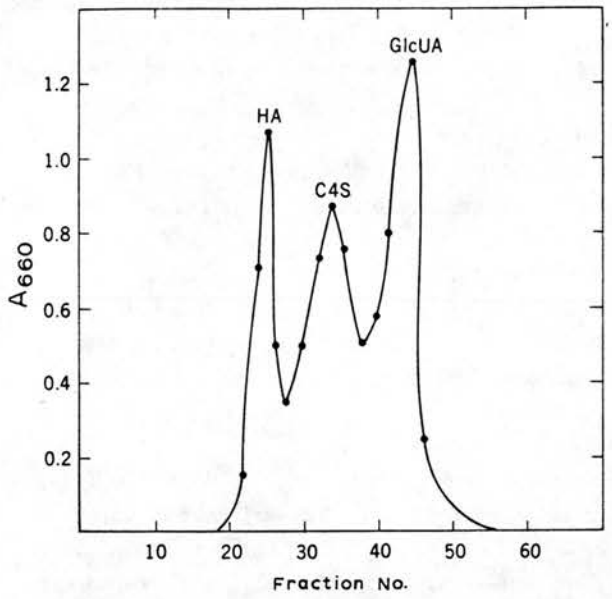


Fig. 2. Elution profile of vitreous humor hyaluronic acid, cartilage chondroitin 4-sulfate, and glucuronic acid (GlcUA) on a controlled-pore, glass-bead column (0.9 × 60 cm). The fractions were analyzed by the orcinol reaction for uronic acid. Abbreviations, see legend to Fig. 1.

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

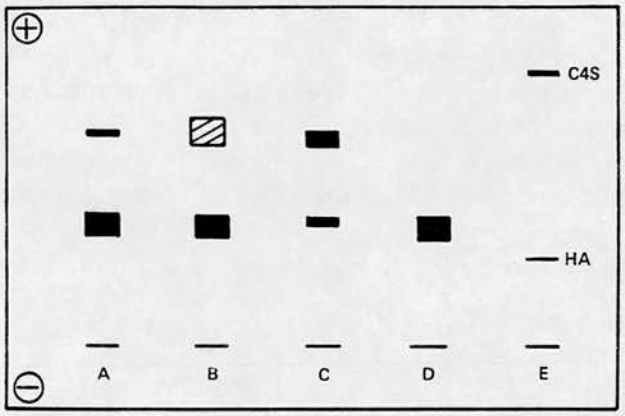


Fig. 3. Cellulose acetate electrophoresis of the intermediate-molecular-weight component of the 0.2M NaCl Fraction obtained by fractionation on a CPG column (Lane A). Conditions for electrophoresis were as described for the 2.0M fraction (see Fig. 1). Lane B, the fraction after treatment with chondroitinase 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 $\beta$-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% $\beta$-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.

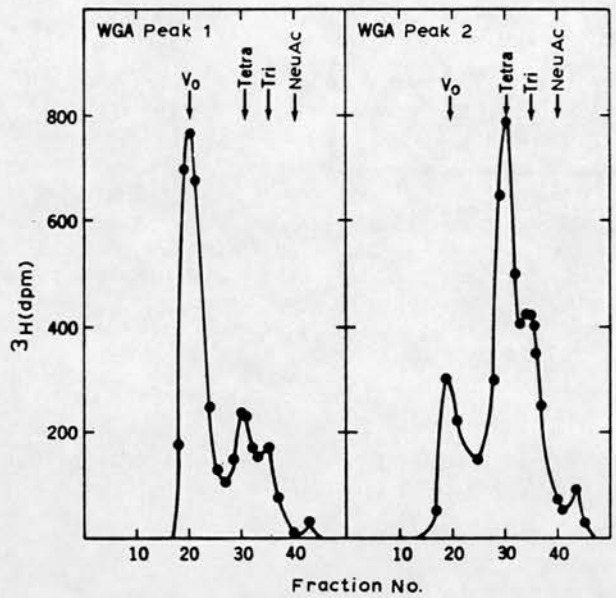


Fig. 4. Chromatography of the WGA–Sepharose subfractions WGA Peak I (material not retained on the WGA–Sepharose column) and WGA Peak II (material retained and eluted with 0.1M *N*-acetyl-glucosamine) of the 0.2M NaCl Fraction on BioGel P-4 column (0.9 × 54 cm), after treatment with alkaline borotritide. 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 $\beta$-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

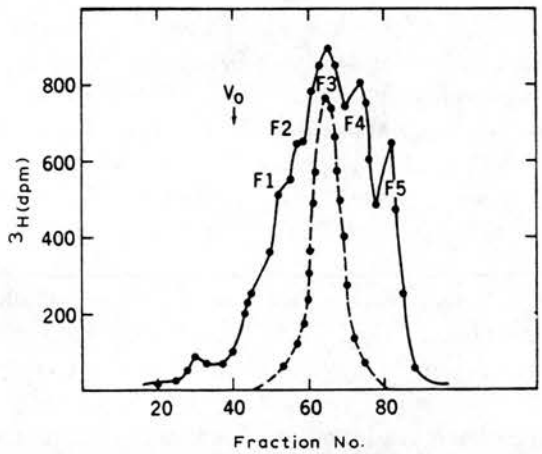


Fig. 5. Elution profiles of the sialoglycoproteins, prepared by solubilization of the tumor with lithium diiodosalicylate, on a Sepharose 4B column. The sialoglycoproteins were labeled with tritium by modification of the sialic acid groups. The solid line illustrates the profile of the whole fraction,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

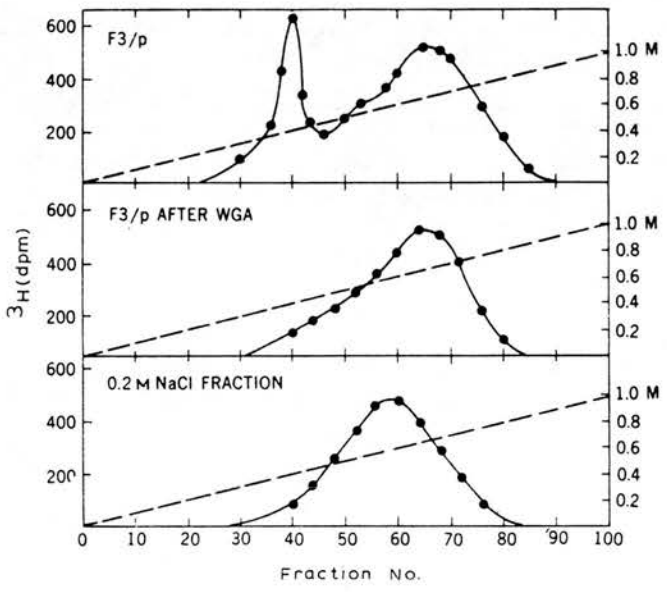


Fig. 6. Chromatography of $^3$H-labeled sialoglycopeptides on a DEAE-cellulose column (1.2 × 40 cm). Elution was performed with a linear gradient of 10mM to M pyridinium acetate, pH 5.2. Upper part: glycopeptides obtained by Pronase digestion of peak F3; middle part: the portion of the glycopeptides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

ACKNOWLEDGMENTS

We are grateful to Mr. John Banks for collecting the tumors, to Mr. Richard Engle for help with the equilibrium ultracentrifugation analysis, and to Mr. Mord Moore for the amino acid analysis. This work was supported in part by Grants CA-15483 and CA-18450 from the National Cancer Institute, National Institutes of Health (DHEW).

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ANALYTICAL BIOCHEMISTRY 91, 186–193 (1978)

# Action of Endo-α-*N*-acetyl-D-galactosaminidase on Synthetic Glycosides Including Chromogenic Substrates

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Received May 3, 1978

The synthetic glycosides, *p*-nitrophenyl- and *o*-nitrophenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-galactopyranosides, were found to be effective chromogenic substrates for an endo-α-*N*-acetyl-D-galactosaminidase. We did not experience any problems when these substrates were used for the screening of column fractions during the purification of the endoenzyme from *Diplococcus pneumoniae* culture filtrates. However, it should be pointed out that a combination of exo-β-galactosidase, capable of cleaving β1 → 3 linkages, and an exo-α-*N*-acetyl galactosaminidase would also liberate nitrophenol from the above substrates. The enzyme had no action on several other synthetic glycosides tested indicating the strict specificity of this enzyme for the disaccharide Gal$\xrightarrow{\beta}$GalNAc linked via an α-linkage to the aglycone. The enzyme was inactive when the aglycone was methanol but shows activity against the glycosides of phenol, nitrophenols, serine, and threonine. The use of *p*-nitrophenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-β-D-galactopyranoside, which is a competitive inhibitor of the endoenzyme, as an affinity ligand for the purification of the enzyme is described.

The purification and characterization of an endo-α-*N*-acetyl-D-galactosaminidase isolated from culture filtrates of *Diplococcus pneumoniae* has been described by us (1,2). Endo and Kobata (3) and Glasgow *et al.* (4) have also described this enzyme activity. In all these studies naturally occurring glycoproteins or glycopeptides derived from them were used as substrates. These substrates are either not readily available (antifreeze glycoprotein) or require extensive isolation procedures (asialofetuin glycopeptide Fraction C). The availability of readily obtainable substrates will greatly facilitate the study of this enzyme as well as the exploration of other sources (both bacterial and mammalian) for the presence of this or similar enzyme activities.

The present report describes the use of synthetic substrates for assaying endo-α-*N*-acetyl-D-galactosaminidase. These studies also provide fur-

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0003-2697/78/0911-0186$02.00/0

TABLE 1

ACTION OF ENDO-α-*N*-ACETYL-D-GALACTOSAMINIDASE ON SYNTHETIC GLYCOSIDES

| Substrate | Relative activity (%) | Reference |
|---|---|---|
| Galβ1 → 3 GalNAc α1 → $OC_6H_4NO_2$ (*p*) | 100 | (5) |
| Galβ1 → 3 GalNAc α1 → $OC_6H_4NO_2$ (*o*) | 100 | (6) |
| Galβ1 → 3 GalNAc α1 → $OC_6H_5$ | 70 | (6) |
| Galβ1 → 3 GalNAc α1 → $OCH_3$ | 0 | (6) |
| Galβ1 → 3 Gal α1 → $OC_6H_4NO_2$ (*p*) | 0 | (6) |
| Galβ1 → 3 GalNAc β1 → $OC_6H_4NO_2$ (*p*) | 0 | (5) |
| Galβ1 → 3 GlcNAc α1 → $OCH_3$ | 0 | (7) |
| Galβ1 → 3 Gal β1 → $OC_6H_4NO_2$ (*p*) | 0 | (6) |
| Galβ1 → 3 GlcNAc β1 → $OC_6H_4NO_2$ (*p*) | 0 | (8) |
| Galβ1 → 6 GlcNAc β1 → $OC_6H_4NO_2$ (*p*) | 0 | (8) |
| GlcNAcβ1 → 6 Gal β1 → $OC_6H_4NO_2$ (*p*) | 0 | (9) |

ther information on the substrate specificity of this enzyme. In addition, the use of a synthetic glycoside as an affinity ligand for the purification of this enzyme was investigated.

## MATERIALS AND METHODS

Partially purified preparations of *D. pneumoniae* glycosidases as well as the inocula of the bacteria were provided by Dr. G. Ashwell. Endoα-*N*-acetyl-D-galactosaminidase was purified from culture filtrates of *D. pneumoniae* by isoelectric focusing as described previously (2).

The following glycosides (see also Table 1) were synthesized as previously described: *p*-nitrophenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α and β-D galactopyranosides (5); *o*-nitrophenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-galactopyranoside (6); methyl- and phenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-galactopyanoside (6); methyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-glucopyranoside (7); *p*-nitrophenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-β-D-glucopyranoside (8); *p*-nitrophenyl-2-acetamido-2-deoxy-6-*O*-β-D-galactopyranosyl-β-D-glucopyranoside (8); *p*-nitrophenyl 6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (9); and *p*-nitrophenyl-3-*O*-β-D-galactopyranosyl-αand β-D-galactopyranosides (6).

Asialofetuin glycopeptide Fraction C (Gal 1 $\xrightarrow{\beta}$ 3 GalNAc $\xrightarrow{\alpha}$ Ser/Thr peptide)[1] was isolated as previously described (2). This glycopeptide was labeled by treatment with galactose oxidase followed by $NaB^3H_4$ reduction according to the method of Morell *et al.* (10). The labeled glycopep-

[1] Abbreviation used: GalNAc, *N*-acetylgalactosamine.

tide was recovered by addition of acetone to destroy excess $NaB^3H_4$ and repeated evaporation with methanol to remove borate. The sample was then passed through a column of AG 50-X2($H^+$); the column was washed with 4 bed vol of 1 N $NH_4OH$ and the washings were evaporated to dryness. Hydrolysis (1 N HCl, 100°C, 6 h) followed by examination of the products by paper chromatography in *n*-butyl acetate:acetic acid: water (3:2:1) showed that 88% of the label was in galactose with the balance in galactosamine.

The action of the endoenzyme on the various synthetic glycosides was determined by incubating 10 μl of a 10 mM solution of the substrate with 20 μl of citrate phosphate buffer, pH 6.2, and enzyme in a total volume of 50 μl at 37°C for various times. In the case of the chromogenic substrates, 450 μl of 0.2 M sodium carbonate was added at the end of the incubation and the absorbance at 420 nm was measured. The hydrolysis of nonchromogenic substrates was checked by the Morgan–Elson reaction (11) in order to detect the released disaccharide. Appropriate controls without enzyme or substrate were also included. The phenol released from the phenylglycoside was determined by using the Folin reagent.

*Preparation of Affinity Columns*

*Method I.* The inhibitor *p*-nitrophenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-β-D-galactopyranoside was reduced with hydrogen using Raney nickel as catalyst (12). The reduced product was isolated and coupled to CH-Sepharose in the presence of 1-ethyl-3-(-3-dimethylaminopropyl)-carbodiimide according to Cuatrecasas (13).

*Method II.* In this method the reduction of *p*-nitrophenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-β-D-galactopyranoside and the coupling of the *p*-aminophenyl compound to cyanogen bromide-activated Sepharose CL6B was carried out as described by Bloch and Burger (14).

## RESULTS AND DISCUSSIONS

The action of the endo-α-*N*-acetyl-D-galactosaminidase on various synthetic substrates is summarized in Table 1. The *p*-nitrophenyl-, *o*-nitrophenyl-, and phenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-galactopyranosides were hydrolyzed by the enzyme. The enzyme was devoid of activity against methyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-galactopyranoside. This is in agreement with the finding that methyl glycosides are generally poor substrates for glycosidases. Previously we have demonstrated the action of the endoenzyme on the *O*-glycoside linkage between the disaccharide (Gal $1 \xrightarrow{\beta} 3$ GalNAc) and serine or threonine residues in glycoproteins (1,2). The inability of the enzyme to hydrolyze any of the other substrates listed in Table 1 further confirms the strict specificity of the enzyme for Gal $1 \xrightarrow{\beta}$ GalNAc linked

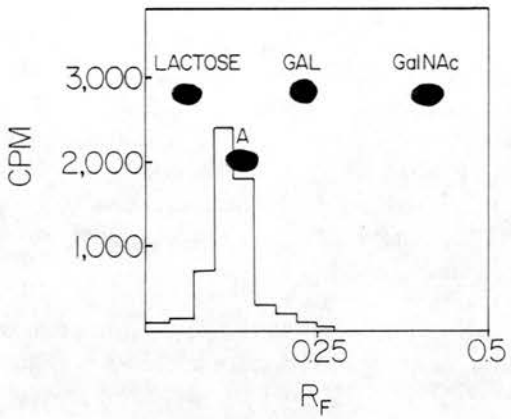


FIG. 1. Paper chromatography of enzyme digests of *o*-nitrophenyl-2-acetamido-2-deoxy-3-*O*-β-galactopyranosyl-α-D-galactopyranoside and [$^3$H]fetuin glycopeptide Fraction C. Paper chromatography was performed on Whatman No. 1 paper using *N*-butylacetate: glacial acetic acid:water (3:2:1) for 24 h. The oligosaccharide from *o*-nitrophenyl substrate, marked A, and the reference sugars were detected by the silver nitrate reagent. The labeled oligosaccharide from [$^3$H]fetuin glycopeptide Fraction C is indicated by the shaded area.

α-*O*-glycosidically to the aglycone (2). Of special interest is the inability of the enzyme to hydrolyze *p*-nitrophenyl-3-*O*-β-D-galactopyranosyl-α-D-galactopyranoside which indicates that the acetamido group at position 2 of galactose is essential for the activity of this enzyme.

The release of *p*-nitrophenol or *o*-nitrophenol from *p*-nitrophenyl- and *o*-nitrophenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-galactopyranosides paralleled color formation in the Morgan–Elson reaction. The product reacting with the Morgan–Elson reagent was identified as Gal $1 \xrightarrow{\beta}$ GalNAc by paper chromatography as illustrated in Fig. 1.

The release of *o*- and *p*-nitrophenols from the chromogenic substrates was linear with respect to time and the proportion of enzyme added. The activity of the enzyme on these substrates at different pH using citrate phosphate buffer is shown in Fig. 2. The optimum pH is in the range 5.5 to 7.0 in the present studies in contrast to an optimum pH of 7.6 for the hydrolysis of asialofetuin glycopeptide Fraction C using Tris/maleate or Tris/HCl buffers reported earlier (2). Endo and Kobata (4) reported a pH optimum of 6.0 in phosphate buffer using asialoglycophorin glycopeptide substrate. Thus, the nature of the substrate, the type of buffer, and the time of incubation appears to influence the optimum pH for the activity of this enzyme. The effects of varying substrate concentrations on the rate of hydrolysis of *p*- and *o*-nitrophenyl substrates were measured and the apparent Michaelis constants ($K_m$) were determined as 0.25 (pH 6.4) and 0.23 mM (pH 6.2), respectively (Fig. 3).

Even though the *p*-nitrophenyl as well as the *o*-nitrophenyl compounds are equally efficiently hydrolyzed by the endoenzyme, the sensitivity of the assay is four fold higher with the former compound due to the higher extinction coefficient of *p*-nitrophenol at 420 nm. The release of the nitrophenol from these substrates would generally indicate the presence of endo-$\alpha$-*N*-acetyl galactosaminidase in the test sample. The only other circumstance which would lead to the release of nitrophenol from the above substrates is the presence of both an exo-$\beta$-galactosidase, capable of acting on Gal 1 $\xrightarrow{\beta}$ 3 GalNAc, and an exo-$\alpha$-*N*-acetyl galactosaminidase. In our studies on *D. pneumoniae* glycosidases we are able to use these chromogenic substrates for detection of the endoenzyme activity in crude culture filtrates and for monitoring column fractions throughout the purification procedure. Exo-$\beta$-galactosidase from *D. pneumoniae* (4), as well as those from Jack Bean (15) and *Clostridium perfringens* (16), either do not hydrolyze or hydrolyze only very slowly $\beta 1 \rightarrow 3$-linked galactosides. However, in order to prove the presence of endo-$\alpha$-*N*-acetyl-D-galactosaminidase activity it will be necessary to examine the products of hydrolysis, for example, by paper chromatography. Detection of the disaccharide, Gal 1 $\xrightarrow{\beta}$ 3 GalNAc, as opposed to monosaccharides galactose and *N*-acetyl galactosamine, would be confirmatory. In conclusion, it should be pointed out that both the *p*-nitrophenyl and the *o*-nitrophenyl compounds are excellent chromogenic substrates for the endo-$\alpha$-*N*-acetyl galactosaminidase and would be useful in further studies on this and similar enzymes.

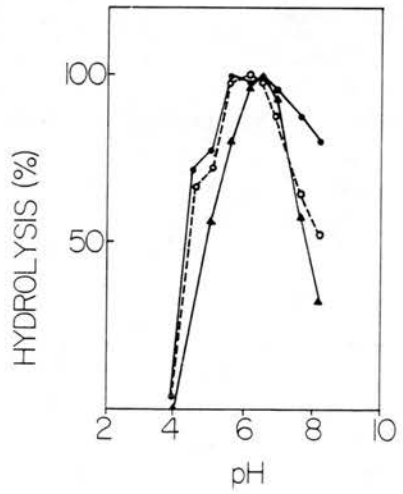


FIG. 2. pH optimum of the endo-$\alpha$-*N*-acetyl-D-galactosaminidase. The enzyme reaction was performed under standard conditions using *p*-nitrophenyl- (●), *o*-nitrophenyl- (○), and phenyl-(▲)-2-acetamido-2-deoxy-3-*O*-$\beta$-galactopyranosyl-$\alpha$-galactopyranoside as substrates.

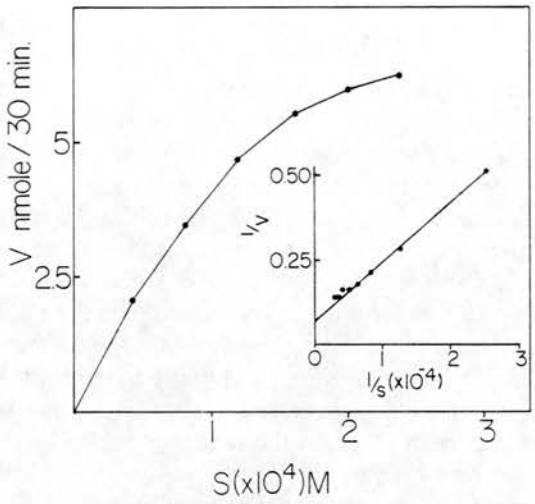


FIG. 3. Effect of *p*-nitrophenyl-2-acetamido-2-deoxy-3-*O*-β-galactopyranosyl-α-galactopyranoside concentration on the rate of reaction. The enzyme reaction was carried out under the standard conditions using citrate phosphate buffer, pH 6.4.

The β-anomer of the *p*-nitrophenyl substrate, *p*-nitrophenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-β-D-galactopyranoside, was found to inhibit the hydrolysis by the endoenzyme of the α-anomer as well as of the asialofetuin Fraction C. A 2 mM concentration of the β-anomer inhibited the hydrolysis of the α-anomer by 90% and that of fetuin glycopeptide by 67%. The inhibition was found to be competitive.

The possibility of using the β-anomer of the *p*-nitrophenyl substrate as an affinity ligand for the endoenzyme was explored. In a representative experiment, a partially purified (2) mixture of the *D. pneumoniae* glycosidases (400 μl) was applied to a column (0.9 × 5 cm) of *p*-aminophenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-β-D-galactopyranoside linked to CH-Sepharose (Method I). The column was washed with 30 ml of 0.01 M citrate buffer pH 6.0 and then eluted with the same buffer containing 0.5 M NaCl. Fractions of 1.5 ml were collected and assayed for the various glycosidases. The results indicated that the endoenzyme was bound to the affinity adsorbent and eluted with NaCl; however, neuraminidase was also bound to the adsorbent and coeluted with the endoenzyme. The binding of the neuraminidase may be due to nonspecific hydrophobic interaction with the spacer arm of CH-Sepharose. We have previously reported (1,2) that both the endoenzyme and the neuraminidase interacted with Affi-Gel 202 which has a spacer arm of about 1.4 nm long. The nonspecific adsorption of neuraminidase was confirmed by chromatography of the mixture of glycosidases on a column of CH-Sepharose. Both the neuraminidase and the endo-α-*N*-acetyl-D-

galactosaminidase bound to CH-Sepharose and were eluted with NaCl. This procedure could be used for the purification of the endoenzyme using gradient elutions with NaCl as in the case of the previously reported chromatography on Affi-Gel 202 (2).

In an attempt to overcome the nonspecific interaction between the spacer arm and neuraminidase, *p*-aminophenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-β-D-galactopyranoside was coupled directly to Sepharose CL6B (Method II). A partially purified fraction of the glycosidases (300 μl) was applied on a column packed with the ligand-Sepharose CL6B. The column was washed with 25 ml of 0.01 M sodium cacodylate buffer, pH 6.0, containing 0.1 M NaCl and then eluted with the same buffer containing 0.5 M NaCl. The results were practically the same as before, namely neuraminidase still bound to the affinity adsorbent in addition to the endoenzyme. The nature of the interaction between neuraminidase and the affinity absorbent is not clear, since there is no hydrophobic spacer arm in this support. It is probable that the hydrophobic *D. pneumoniae* neuraminidase is interacting with the phenyl group of the ligand. These results suggest that a one-step affinity chromatography purification of the endoenzyme may not be feasible due to the apparently high hydrophobicity of the *D. pneumoniae* neuraminidase. However, if the endoenzyme is first partially purified free of neuraminidase by, for example, chromatography on a column of DEAE-Sephadex as described by Glasgow *et al.* (4) further purification could be carried out by using this affinity adsorbent. The synthetic ligand, *p*-aminophenyl-2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-β-D-galactopyranoside is a good alternative to antifreeze glycoprotein for the preparation of affinity adsorbent (4). An affinity adsorbent prepared from this ligand could be repeatedly used, unlike the adsorbent prepared from the substrate antifreeze glycoprotein, which on repeated use could loose activity due to hydrolysis of the ligand from the Sepharose.

## ACKNOWLEDGMENTS

This work was supported by United States Public Health Service Grants CA 15483 (to E. A. Davidson) and CA 17686 (to V. P. B.) and by Biomedical Research Grant RR5648-11 (to K. L. M.). We are grateful to Dr. E. A. Davidson for his encouragement and support and to Dr. G. Ashwell for providing the *Diplococcus pneumoniae*.

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# Purification, Properties, and Analysis of Human Asthmatic Bronchial Mucin†

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ABSTRACT: A high molecular weight, mucin-type glycoprotein has been isolated from a sample of human bronchial secretion obtained from an asthmatic patient. The glycoprotein elutes in the void volume of a Sepharose 4B column, and its mobility is unchanged in the presence of dithiothreitol. Examination of the material in the analytical ultracentrifuge under equilibrium conditions gave an estimated minimal molecular weight of $1.8 \times 10^6$ with aggregation to $10 \times 10^6$ or greater. Analysis showed the predominant amino acids to be serine, threonine, and proline with a low content of methionine and cysteine; glucosamine and galactosamine were present in approximately equimolar amounts and comprised 28% by weight of the glycoprotein. Composition analysis after alkaline borohydride treatment showed that the saccharide chains were O-glycosidically linked through *N*-acetylgalactosamine to both serine and threonine residues in the peptide backbone. Carbohydrate analysis by gas–liquid chromatography identified galactose, fucose, glucosamine, galactosamine, and sialic acid in an approximate molar ratio of 3:3:2:2:1. The sialic acid is present as *N*-acetylneuraminic acid. A portion (7%) of the saccharides are present as galactosyl→*N*-acetylgalactosaminyl residues linked to the protein core. A glycopeptide fraction was isolated following pronase digestion and had a molecular weight of $1.5 \times 10^5$. This value was not significantly changed by either removal of sialic acid or exposure to guanidinium chloride. These data support the presence of large clusters of oligosaccharides which are covalently linked to the serine and threonine residues of the peptide.

Human mucosal epithelia from all sources are coated with mucus. The specific properties of mucus vary among organ tracts (respiratory, salivary, gastric, and cervical), but the overall chemical and physical similarities reflect a common lubricative and protective function. The respiratory mucus has the additional specific roles of particulate clearance and maintenance of proper water balance in the tracheobronchial tract.

The chemical composition of unfractionated human bronchial secretions from healthy and diseased states has been generally defined as to total inorganic, protein, lipid, and carbohydrate content (Basch et al., 1941; Chernick & Barbero, 1959; Matthews et al., 1963; Masson et al., 1965; Schultze & Heremans, 1966). Physical properties, such as viscosity and elasticity, have also been examined (Denton, 1960).

An understanding of the relationship between the macromolecular components of respiratory mucus and the physiological functions of this secretory product requires, in part, isolation and characterization of the individual glycoprotein components.

In general, fractionation of mucus by gel exclusion chromatography on Sephadex G-200 or Sepharose 4B produces two major peaks. The high molecular weight excluded component, commonly referred to as mucin or epithelial glycoprotein, is carbohydrate rich and is apparently responsible for the physicochemical structure and rheological properties of the mucus secretion (Havez et al., 1967). The majority of studies on the glycoprotein component of bronchial secretion have been histochemical and autoradiographic (Havez et al., 1967; Lamb & Reid, 1969; Sturgess & Reid, 1972). The chemical composition of some preparations has been described (Werner, 1953; Havez et al., 1968; Roussel et al., 1972, 1975; Lamblin et al., 1973; Roberts, 1974; Boat et al., 1976; Lafitte et al., 1977). Bhattacharyya and Lynn have reported on the saccharide structure of a glycoprotein isolated from the alveoli of patients with alveolar proteinosis (Bhattacharyya & Lynn, 1977; Bhattacharyya et al., 1976). The carbohydrate in this macromolecule, however, is of the asparagine-linked, mannose-containing type and appears unrelated to the predominant mucin glycoprotein. This paper deals with the purification, characterization, and partial structural identification of the major macromolecular component of bronchial secretion. There are considerable technical difficulties in collecting adequate amounts of normal human bronchial secretion. Accordingly, the high molecular weight glycoprotein component discussed in this report was isolated from the tracheobronchial secretions of an asthmatic patient. Separate studies in our laboratory have demonstrated that organ cultures of human trachea produce a mucin glycoprotein with physical and chemical properties similar to if not identical with those described for the bronchial secretion product. In addition, preliminary analyses of aspirate mucus from pediatric patients show the presence of comparable components. Thus, this study should provide background data for comparison with the structural features of bronchial mucins obtained from healthy individuals and from individuals with defined pulmonary pathology. It should be noted that mucus from patients with cystic fibrosis has been extensively investigated but cannot be considered representative of normal material (Roussel et al., 1975).

## Experimental Procedures

*Materials.* Human bronchial secretion obtained by a 4-day collection (100 mL) from an asthmatic patient was generously provided by Dr. William Lynn, Department of Biochemistry, Duke University. The patient was a secretor and the crude mucus sample had blood group A activity (Morgan & Watkins, 1951). The crude mucin was examined for microbial contamination by culturing an aliquot on growth medium. No microorganisms were observed from this sample or subsequent

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[Reprinted from Biochemistry, (1979) 18, 2430.]

collections from the same patient.

The secretion was promptly frozen and maintained in the frozen state until workup. The material was thawed, diluted 1 to 10 with $H_2O$, and adjusted to 5 mM $NaN_3$ to prevent microbial contamination during subsequent procedures. The diluted sample was stirred overnight at 4 °C and centrifuged at 7000*g* for 20 min to remove a small amount of cellular debris. There was no visible gel-like material present in the pellet. The pellet contained less than 10% by weight of the starting material was not examined further. The supernatant, which contained over 90% of the carbohydrate content of the original material, was applied to a 5 × 92 cm column of Sepharose 4B (Pharmacia Fine Chemicals). Chromatography was performed at pH 5.0 as indicated in Figure 1. The high molecular weight void volume component, identified as the mucin glycoprotein, was dialyzed against $H_2O$ at 4 °C and lyophilized (150 mg).

*Analytical Methods.* Analyses for the carbohydrate components of glycoproteins were carried out as follows: chromatographic column effluents were screened for neutral sugar by the phenol–sulfuric acid method (Dubois et al., 1956) with galactose as a standard, and for sialic acid by the thiobarbiturate procedure (Aminoff, 1961) with *N*-acetylneuraminic acid as a standard. Specific sugar components were identified by gas–liquid chromatography of alditol acetates (Sawardekar et al., 1965; Lehnhardt & Winzler, 1968) following mineral acid hydrolysis or by methanolysis of the sugars followed by conversion to the trimethyl silyl ethers (Clamp et al., 1972) prior to gas–liquid chromatography; the latter procedure only was employed for sialic acid. Glucosamine and galactosamine were also determined on the amino acid analyzer after acid hydrolysis in vacuo at 100 °C for 8 h in 4 N HCl; galactosaminitol was determined on the amino acid analyzer after acid hydrolysis as for the hexosamine analyses (Cheng & Boat, 1978). Asialoglycoprotein and asialoglycopeptides were prepared by incubation of the appropriate material at 37 °C with *Vibrio cholerae* sialidase (protease free) (Calbiochem). The incubation was carried out in 0.1 M sodium acetate, pH 5.6, containing 1 mM $CaCl_2$ and the material then was chromatographed on Sepharose 4B by utilizing 0.1 M acetate buffer, pH 5.0. The fractions containing sialic acid were combined and the identity of the released product as *N*-acetylneuraminic acid confirmed by comparison with authentic standards employing descending paper chromatography on Whatman no. 1 paper with *N*-butyl acetate:glacial acetic acid:$H_2O$ (3:2:1) as solvent. In general, exclusion columns were calibrated with blue dextran (V) or [$^{14}$C]glucose (S).

Protein was determined by the procedure of Lowry (Lowry et al., 1951) or when screening chromatographic column effluents by a method employing Coomassie Brilliant Blue G (Sigma Chemical Co.) (Bradford, 1976). Ninhydrin was used to assay for free amino groups following Pronase digestion. Amino acid analyses were performed by ion-exchange chromatography with a Beckman Model 120C or 121 amino acid analyzer following hydrolysis of the samples in vacuo for 24 h at 110 °C with 6 N HCl.

*Reduction and S-Carboxymethylation.* Reduction and S-carboxymethylation of the glycoprotein were performed according to a modification of the method of Crestfield (Crestfield et al., 1963). The glycoprotein (2 mg) was dissolved in 1.4 M Tris-HCl, pH 8.6 (10 mL), containing 10 mM EDTA, 5.7 M guanidinium chloride, and 0.2 M DTT. The samples were incubated at 25 °C for 4 h and the reduced glycoprotein was reacted with iodoacetic acid (0.25 g) and 2 M NaOH (200 μL) for 45 min at room temperature in the dark. The reaction mixtures were then extensively dialyzed at 4 °C in the dark against $H_2O$.

*Pronase Digestion.* Digestion of 5 mg of glycoprotein in 1.0 mL of 0.1 M borate buffer, pH 8.0, containing 0.01 M calcium acetate was carried out at 37 °C for 72 h with the addition of 0.5 mg of Pronase (Calbiochem)/10 mg of glycoprotein at times zero, 24, and 48 h. A trace amount of toluene was added to prevent microbial contamination. The Pronase digestion was also carried out in 50 mM Tris buffer, pH 8.0, 0.01 M calcium acetate and in an independent digestion, Proteinase K (EM Biochemicals) (Ebeling et al., 1974) was used in place of Pronase. Exclusion chromatography of the protease digest was initially performed at either pH 5.2 in 0.1 M pyridine acetate with Sephadex G-25 (Pharmacia Fine Chemicals) or at pH 5.0 in 0.1 M acetate buffer by utilizing Sepharose 4B. The void volume fraction of the G-25 column was assayed for blood group activity (Morgan & Watkins, 1951). Anion-exchange chromatography was performed by using a pyridine acetate gradient on DEAE-cellulose (Whatman Biochemicals, Ltd.).

*Electrophoretic Studies.* Polyacrylamide gel electrophoresis was performed on 6% polyacrylamide gels in the presence of sodium dodecyl sulfate ± β-mercaptoethanol at 7 V/cm of gel (Weber et al., 1972). Samples (15–20 μg) were incubated in $NaDodSO_4$ ± BME for 2 h at 40 °C and then applied to the gels. Electrophoresis was also performed with gels containing 0.5% agarose (Bio-Rad Laboratories) and 1.5% acrylamide (Bio-Rad Laboratories) at 2.5 mA/tube for 2 h (Holden et al., 1971). Samples of 200–500 μg were applied to the agarose–acrylamide gels. Gels were stained for protein with Coomassie Brilliant Blue R (Sigma Chemical Co.) and for carbohydrate by the periodate–Schiff method (Zacharius et al., 1969).

*Sedimentation Equilibrium Studies.* The glycoprotein and the pronase resistant glycopeptide core were examined by analytical equilibrium ultracentrifugation employing the meniscus depletion method (Yphantis, 1964). Details of the conditions used are given in the appropriate figure legend. Prior to centrifugation, the sample in buffer was dialyzed for 24 h against the same buffer. The partial specific volume was not explicitly measured for this glycoprotein. Based on the amino acid analysis and carbohydrate composition, a figure of 0.60 was estimated; the volume for the glycopeptide was estimated to be 0.54.

*Circular Dichroism.* Spectra were recorded at 23 °C from 300 to 205 nm with a Cary Model 60 recording spectropolarimeter equipped with a CD attachment. The glycoprotein or glycopeptide was dissolved at a concentration of 1 mg/mL in 0.1 M NaCl or $H_2O$, respectively. The spectrum of the solvent was recorded before and after each experiment to correct for any base-line drift.

*Alkaline Borohydride Treatment.* A solution of 4 mg of glycoprotein in 2 mL of 0.05 M KOH–1.0 M $NaBH_4$ was incubated for 72 h at 37 °C (Carlson, 1966). The excess borohydride was eliminated by adjusting the pH to 4.5 at 4 °C by addition of 4 M acetic acid. The solution was evaporated three times with methanol and the resulting material examined by chromatography on a P10 column (Bio-Rad Laboratories) in 0.1 M pyridine–acetate buffer, pH 5.2. In some experiments, $NaB^3H_4$ (NEN) was employed to permit labeling of the oligosaccharide chains.

*endo-α-Acetylgalactosaminidase Digestion of the Glycoprotein.* Treatment of 2.0 mg of the desialylated glycoprotein

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; BME, β-mercaptoethanol; PAS, periodic acid Schiff; DDT, dithiotreitol.

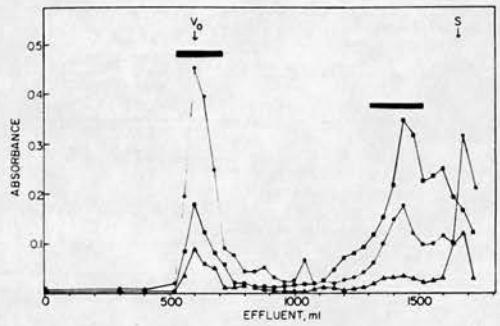



FIGURE 1: Sepharose 4B chromatography of the supernatant from the diluted human bronchial secretion. The column (5 × 92 cm) was operated with a pressure head of 50 cm at a flow rate of 100 mL/h. The eluting buffer was 0.1 M sodium acetate, pH 5.0. Representative aliquots were analyzed for neutral sugar (●), protein (■), and sialic acid (▲). Absorbance data are at 490, 660, and 550 nm, respectively. The bars show the fractions that were combined for further study.

Table I: Amino Acid Analysis of Bronchial Secretion after Chromatography on Sepharose 4B

| amino acid | residues thousand fraction I | residues fraction II | glycopeptide (Pronase resistant core) |
|---|---|---|---|
| Lys | 29 | 64 | 17 |
| His | 21 | 22 | 26 |
| Arg | 40 | 40 | 28 |
| Asp | 70 | 115 | 35 |
| Thr | 170 | 59 | 302 |
| Ser | 110 | 59 | 186 |
| Glu | 78 | 138 | 44 |
| Pro | 103 | 79 | 105 |
| Gly | 72 | 63 | 72 |
| Ala | 86 | 75 | 92 |
| $^1/_2$-cystine | 15 | 35 | trace |
| Val | 69 | 66 | 33 |
| Met | 10 | 11 | |
| Ile | 29 | 22 | 9 |
| Leu | 63 | 85 | 36 |
| Tyr | 11 | 27 | 6 |
| Phe | 21 | 41 | 9 |
| glucosamine[a] | 14.4% | 5.1% | 19.1% |
| galactosamine[a] | 13.3% | 1.0% | 17.7% |

[a] Data are expressed as percent by weight of the total glycoprotein.

with an *endo-α-N*-acetylgalactosaminidase isolated from *Diplococcus pneumoniae* (Bhavanandan et al., 1976) was carried out at 37 °C for 24 h in 1 mL of 0.1 M Tris–maleate buffer, pH 7.6. After the incubation, aliquots of the mixture were assayed for Morgan–Elson positive material by using authentic Galβ1→3GalNAc as standard (Reissig et al., 1955). The released oligosaccharide product was further identified by paper chromatography on Whatman no. 1 paper by using *N*-butyl acetate:glacial acetic acid:$H_2O$ (3:2:1) as a solvent.

*End-Group Analysis.* Amino-terminal end-group analysis was performed by utilizing dansyl chloride (Gray, 1967). Following hydrolysis with 6 N HCl for 18 h, the products were examined by two-dimensional thin-layer chromatography on polyamide sheets (Woods & Wang, 1967).

## Results

*Purification and Physical Properties of the Mucin-Type Glycoprotein.* After the low-speed centrifugation (7000*g*) of the bronchial secretion, 93% of the neutral sugar and 88% of the sialic acid (thiobarbituric acid analysis) remained in the supernatant. This supernatant was analyzed by exclusion chromatography on a Sepharose 4B column. The mucin-type glycoprotein emerged in the void volume (Figure 1) which also contained nearly all of the blood group A activity. The amino acid analysis of the void volume (500–700 mL) is presented in Table I; the total amino sugar content comprised 28% by weight of the glycoprotein; sulfate content was 4.5%. Fraction II (1300–1500 mL), when analyzed for amino acids, gave a profile more characteristic of serum-type glycoproteins (Table I). In addition, the presence of mannose, alkali stability of the peptide–oligosaccharide linkage, and extensive reactivity with anti-human serum following immunoelectrophoresis identified the components present as primarily circulating glycoproteins. The possibility exists that lung cell specified macromolecules are also to be found in this mixture. However, this fraction was not further studied.

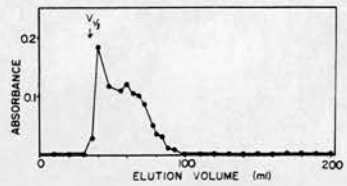



FIGURE 2: Bio-Gel A-150m chromatography of human bronchial mucin (Sepharose 4B-pk I, Figure 1). The column (1.5 × 55 cm) was operated in 0.1 M sodium acetate buffer, pH 5.0, at a pressure head of 30 cm and a flow rate of 9 mL/h. The column fractions were assayed for neutral sugar; absorbance data are at 490 nm.

Sepharose 4B gel filtration of the mucin-type glycoprotein preincubated with DTT and run in its presence showed no change in the elution pattern. This result suggests that reduction of disulfide bridges does not lead to a large decrease in molecular size. The peak I material from the Sepharose 4B column was subsequently chromatographed on a Bio-Gel A-150 M (Bio-Rad Laboratories) column (1.5 × 55 cm) by utilizing pH 5.0, 0.1 M acetate buffer. The elution pattern showed some retention of the glycoprotein (Figure 2), but substantial material was still present in the void volume. The heterogeneous profile seen in the Bio-Gel A-150 M column elution pattern may represent aggregation, entrapment, peptide and/or saccharide polydispersity, or some combination of these.

End-group analysis of fraction I revealed no measurable amino-terminal residue. This result is compatible with the properties of some glycoproteins where the N-terminal amino acid is found to be blocked, e.g., $\alpha_1$-acid glycoprotein (Schmid, 1954). Additionally, based on the amount of material employed and the detection sensitivity of the analytical technique, the results indicate the absence of significant contamination by proteins with accessible termini.

The molecular weight of fraction I was examined by equilibrium sedimentation ultracentrifugation at three different concentrations in 0.5 M NaCl (Figure 3). Employing a partial specific volume of 0.60, a minimum molecular weight of $1.8 \times 10^6$ can be estimated, although variation in the frequency of saccharide chains as well as their nature introduces an uncertain level of heterogeneity. The upward displacement of the plot of $\sigma_w$ vs. concentration is indicative of the presence of species of higher molecular weight. An evaluation of the data by means of a two species plot (Roark & Yphantis, 1969) gave no indication of specific association stoichiometry, although components with $M_w$ of $10^7$ or higher are apparently present. These are likely to be aggregates but may arise in

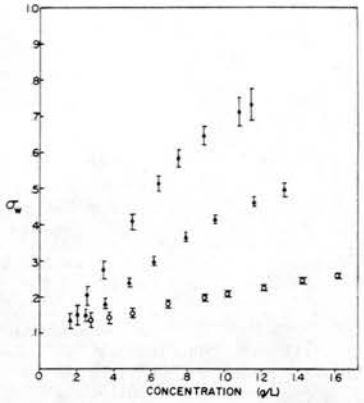



FIGURE 3: Equilibrium sedimentation analyses of the void volume peak described in Figure 1. The sample was run at 20 °C, 2000 rpm, and at three different loading concentrations: 0.25 (●), 0.5 (▲), and 1.0 mg/mL (O). Data were obtained 8 h after equilibrium was reached, 36 h after the initiation of the run. The molecular weight parameters were calculated by a computer program from fringe displacement patterns obtained after equilibrium was reached.

part from the heterogeneity present in the minimal species.

The mucin-type glycoprotein fraction recovered from the Sepharose 4B chromatography was completely excluded from 6% polyacrylamide gels when analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (± β-mercaptoethanol). These results combined with those from the ultracentrifugation study indicated a high "native" molecular weight. The limitations of this type of molecular weight estimation, however, preclude any statements regarding the role of disulfide bonds in the macromolecule. Accordingly, the glycoprotein was reduced with dithiothreitol in the presence of guanidinium chloride and any –SH groups generated by reduction were carboxymethylated with iodoacetamide. The bulk of the material, approximately 80%, still appeared at the void volume of a Sepharose 4B column, although a minor component may be slightly included. The native and reduced–carboxymethylated glycoproteins were further examined by electrophoresis in 0.5% agarose–1.5% polyacrylamide gels. The native glycoprotein did not enter this gel and no lower molecular weight protein or carbohydrate components were detected after loading the gels with 500 μg of glycoprotein. The reduced glycoprotein showed two components corresponding in proportion to those observed on Sepharose 4B chromatography. The included component stained weakly, but detectably, with the periodic acid–Schiff reagent and is presumably a glycoprotein.

*Chemical Composition.* The carbohydrate analysis of the material in the void volume of the 4B column is presented in Table II. The overall carbohydrate content was estimated to represent 70% by weight of the glycoprotein. The sialic acid was identified by paper chromatography as *N*-acetylneuraminic acid and was completely susceptible to *Vibrio cholerae* neuraminidase. The fact that mannose was absent indicated that the material was free of glycoproteins wherein the carbohydrate moiety is attached via the amide nitrogen of asparagine.

The linkage of the oligosaccharides to the peptide core was concluded to be O-glycosidic based on the results of treatment with alkaline borohydride. The data indicate that two-thirds of both the serine and threonine residues are substituted. Chromatography of the reaction products on a P-10 column

Table II: Carbohydrate Composition of the Glycoprotein before and after Alkaline Borohydride Treatment

| carbohydrate residue | molar ratio[a] glycoprotein | molar ratio[a] $OH-NaBH_4$ treated |
|---|---|---|
| L-fucose | 2.6 | 2.9 |
| D-galactose | 3.1 | 2.7 |
| *N*-acetyl-D-galactosamine | 1.4 | 0.2 |
| *N*-acetyl-D-glucosamine | 2.0 | 1.8 |
| sialic acid | 1.0 | 1.0 |
| *N*-acetyl-D-galactosaminitol | 0.0 | 1.0 |

[a] Relative to sialic acid.

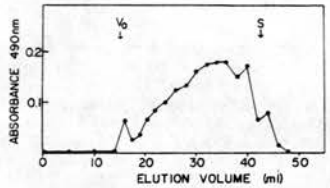



FIGURE 4: Bio-Gel P-10 chromatography of bronchial secretion (Sepharose 4B-pk I, Figure 1) which has been treated with $NaBH_4$–KOH. The column (1 × 50 cm) was run in 0.1 M pyridine–acetate buffer at a flow rate of 8 mL/h. Fractions were assayed for neutral sugar.

showed that essentially all of the neutral sugar was included (Figure 4); the peptide product of this reaction was not studied. Based on the chromatographic mobility of known oligosaccharides, the size of the eliminated products ranged from 2 to about 9 monosaccharides with the majority containing 5 to 7 sugars. About 85% of the *N*-acetylgalactosamine was reduced to galactosaminitol, indicating that GalNAc is the linkage sugar (Table II). The stoichiometry of the monosaccharides, the blood group A reactivity of the starting glycoprotein, and the heterogeneity shown on P-10 chromatography clearly indicate that several types of oligosaccharides are present.

*Purification and Physical and Chemical Characterization of the Glycopeptides.* Preliminary fractionation of the Pronase digest was performed on a Sephadex G-25 column. Over 90% of the material which was positive for neutral sugar eluted at the void volume and accounted for virtually all (96%) of the starting carbohydrate content. The column fractions contained no detectable protein based on the colorimetric procedure, although amino acids and small peptides were readily detected in a second peak eluting near the included volume. The glycopeptide material was excluded from both G-50 and controlled pore glass (10–240) columns. However, it was included on a Sepharose 4B column in sharp contrast to the starting material (Figure 5). The void volume fraction of the G-25 column was found to exhibit blood group A activity. This result was expected since the A determinants are in the carbohydrate structure. Quantitation of the A activity was not attempted on this fraction.

A second Pronase digestion performed in Tris buffer or digestion with proteinase K yielded material which failed to enter the G-25 gels but was included on Sepharose 4B. Incubation of the Pronase digest with dithiothreitol and chromatography on Sepharose 4B in the presence of dithiothreitol did not change the profile of carbohydrate reactive material.

The amino acid composition of the glycopeptide (Table I) shows a significant enrichment in threonine and serine (nearly 50% of the total), indicating that the large molecular weight of this fragment is due to clusters of serine and threonine

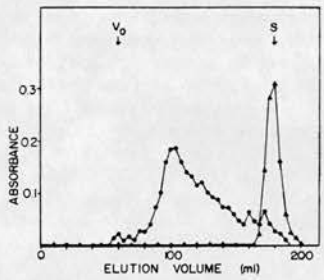



FIGURE 5: Sepharose 4B chromatography of a 72-h Pronase digest of human bronchial secretion (Sepharose 4B-pk I, Figure 1). The column (2 × 66 cm) was operated at a pressure head of 50 cm at a flow rate of 15 mL/h with 0.1 M sodium acetate buffer, pH 5.0. Fractions were analyzed for neutral sugar (●) and free amino groups (▲). Absorbance data are at 490 and 570 nm, respectively.

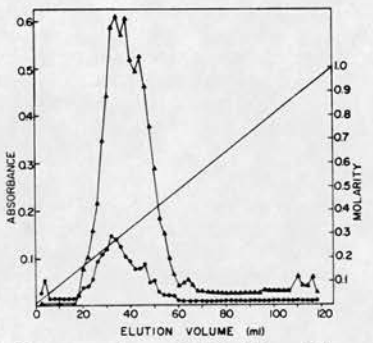



FIGURE 6: Chromatography on DEAE-cellulose of the pooled void volume fractions from G-25 chromatography of the Pronase digested material (Figure 5). The material was eluted with a linear gradient from 10 mM to 1 M pyridine–acetate, pH 5.2. Fractions were analyzed for neutral sugar (●) and sialic acid (▲). Absorbance data are at 490 and 550 nm, respectively.

residues protected from further proteolytic digestion by carbohydrate side chains.

The Pronase digestion material from the void volume of the G-25 was pooled, concentrated, and run on a DEAE-cellulose column. The material was eluted with a linear gradient from 10 mM to 1 M pyridine–acetate, pH 5.2. As illustrated in Figure 6, all of the material was eluted between 0.2 and 0.5 M pyridine–acetate but no clear fractionation was obtained. This result suggests that a distinction between sulfated and nonsulfated components may not be present.

$NaDodSO_4$–polyacrylamide gel electrophoresis (10 cm; 6% polyacrylamide gels ± $\beta$-mercaptoethanol) was carried out with the Pronase digested material (void volume of the G-25). There was no detectable protein (Coomassie Blue stain) but two bands contained carbohydrate and reacted with the PAS reagent. One of the bands was comprised of material which did not enter the gel, and the second band migrated about 1 cm. The results were identical whether or not $\beta$-mercaptoethanol was present.

Since the high carbohydrate content of glycopeptides makes molecular weight estimation unreliable by gel electrophoretic methods, the glycopeptide obtained after Pronase digestion was examined by ultracentrifugation. Fractions from the major peak of the Sepharose 4B chromatography of the unreduced glycopeptides were combined, dialyzed, lyophilized, resuspended in 50 mM Tris, pH 8.0, or 50 mM Tris, pH 8.0,

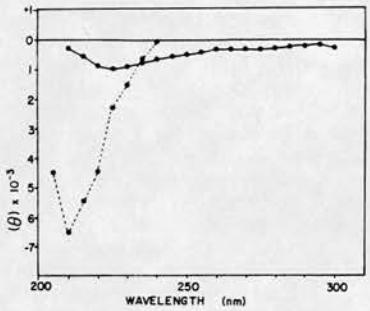



FIGURE 7: Circular dichroic spectrum of intact bronchial secretion (Sepharose 4B-pk I, Figure 1) (—) compared with the spectrum of the glycopeptide (---) core after Pronase digestion. Concentration was 1 mg/mL.

+ 4 M guanidinium chloride, and dialyzed against the appropriate buffer. This material was examined in the analytical ultracentrifuge under equilibrium conditions. Based on a partial specific volume of 0.54, the estimated molecular weight for the glycopeptides was $1.5 \times 10^5$; there was negligible polydispersity.

In an attempt to eliminate any aggregation of the glycopeptides which might be due to terminal sialyl residues, the Pronase digest was treated with sialidase and rechromatographed on Sepharose 4B. All detectable sialic acid was removed from the glycopeptides, but the elution profile was essentially unchanged. An ultracentrifugation study of the major glycopeptide peak following sialidase digestion gave an estimated molecular weight of $1.4 \times 10^5$, a value only slightly less than that for the starting glycopeptides. A further centrifugation study of the desialylated glycopeptide in the presence of 4 M guanidinium chloride gave similar results.

The conformation of the glycoprotein and the Pronase core were examined by circular dichroic measurements. The spectrum (Figure 7) for the intact glycoprotein shows a negative absorption trough at 225 nm; the glycopeptide obtained after Pronase digestion showed a shift in the absorption trough to 210 nm.

## Discussion

Chemical characterization of the bronchial glycoproteins present in gel-like secretions requires a solubilization step prior to analysis. In order to prepare material as nearly native as possible, a mild procedure of isolation was used and neither detergent nor reducing agent was employed. Since nearly all of the carbohydrate was recovered in the soluble fraction, it may be concluded that the analytical data obtained are representative. Following solubilization, chromatography on Sepharose 4B separated the mucin type glycoprotein from lower molecular weight protein components. This was substantiated by the absence of lower molecular weight proteins upon $NaDodSO_4$ gel electrophoresis (±BME) of the mucin in 6% polyacrylamide gels. These data combined with the agarose–acrylamide electrophoresis results support the minimum molecular weight of $1.8 \times 10^6$ calculated for the mucin glycoprotein from the sedimentation equilibrium study.

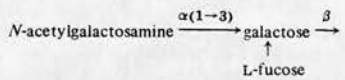
Hill et al. have proposed aggregation of subunits to account for the high molecular weight of native and asialo ovine submaxillary mucin. They attribute the aggregation to noncovalent interactions dependent upon the carbohydrate moieties of the mucin (Hill et al., 1977). The presence of disulfide bonds in bronchial mucin has also been suggested

by several investigators (Boat et al., 1976; Roberts, 1976; Creeth et al., 1977). The agarose–acrylamide electrophoresis indicates that a small portion (approximately 20%) of the native material is associated via disulfide bonds. However, the minimal size of the reduced product is estimated at $1 \times 10^6$, based on gel electrophoresis studies with submaxillary mucins (Holden et al., 1971).

An amino acid analysis of the mucin showed that serine and threonine comprise about one-third of the residues and together serine, threonine, proline, glycine, and alanine constitute more than 50% of the total amino acid content. These analytical data are somewhat lower than those reported for other mucins or from bronchial glycoproteins obtained from a patient with cystic fibrosis (Roussel et al., 1975). In the latter case, the amino acid composition was comparable to that of the glycopeptide obtained after Pronase digestion in this study. The carbohydrates galactose, fucose, glucosamine, galactosamine, and sialic acid are present in a molar ratio of 3:3:2:2:1. Sulfate is presumably esterified to the galactose moieties (Havez et al., 1968; Roussel et al., 1972, 1975; Boat et al., 1976) but detailed localization of the sulfate ester groups was not achieved in this study. Mannose and uronic acid which are constituents of serum glycoproteins and proteoglycans, respectively, were not present. The mucin was found to exhibit blood group A activity (Watkins, 1972). A number of additional bronchial mucus samples have been obtained from pediatric patients hospitalized for surgery or other reasons not involving the pulmonary system. These have all reflected the blood group status of the donor and presumably are characteristic epithelial cell products of secretors.

The carbohydrate–protein linkages were shown by $\beta$ elimination to involve *N*-acetyl-D-galactosamine attached to serine and threonine via *O*-glycosyl bonds. Approximately two-thirds of the serine and threonine residues were substituted; thus approximately one of every five amino acids has a saccharide chain attached. The structures of the carbohydrate prosthetic groups have not been fully defined but the results of the P-10 chromatography indicate a heterogeneous size distribution (2–9 sugars). A small portion (7%) of the oligosaccharides are present as galactosyl-$\beta$1→3-*N*-acetylgalactosaminyl disaccharides linked to the protein core through serine and threonine residues. This conclusion is based on the results of digestion of the glycoprotein with an *endo*-$\alpha$-*N*-acetylgalactosaminidase from *Diplococcus pneumoniae* (Umemoto et al., 1977). Since the enzyme preparation employed was free of sialidase, *exo*-acetylhexosaminidase, galactosidase, and fucosidase activities and acts only on unsubstituted disaccharidyl substrates, it may be inferred that this structural entity arises from incomplete chains. The Gal→GalNAc disaccharide is commonly present in mucin type glycoproteins.

Due to the blood group A activity, some of the oligosaccharides should have the sequence




at their nonreducing end.

The results of the Pronase digestion indicate that the entire carbohydrate content is localized in a serine–threonine rich region of the polypeptide. It may be estimated that the polypeptide moiety of the glycopeptide obtained after Pronase digestion contains about 100 amino acids of which about half are substituted with saccharide chains. Reconciling these data with the molecular weight estimates of the native mucin requires either noncovalent associative behavior of the saccharide-free domains or the presence of several sections of high sugar content and spacer regions completely free of carbohydrate; composite structures are also feasible. Glycoprotein structural models composed of two types of regions have been proposed for blood group substances (Dunstone & Morgan, 1965; Kristiansen & Porath, 1968; Donald, 1973; Goodwin & Watkins, 1974) and for the epithelial secretions of bovine (Bhushana Rao & Masson, 1977) and human (Roberts, 1976) origin. Additionally, the clustering of serine and threonine amino acid residues has been observed in the regions of the blood group substances carrying the carbohydrate chains (Goodwin & Watkins, 1974).

The high molecular weight of the glycopeptide fraction is not due to aggregation caused by the presence of the negatively charged terminal sialic acid residues, to ionic interactions or to hydrogen bonding.

A distinctive characteristic of the circular dichroic spectra of both the intact mucin and the glycopeptide fraction was a negative trough at 210 mM for the glycopeptide fraction, similar to the results reported for milk oligosaccharides (Kabat et al., 1969). The intensity of the negative absorption observed after Pronase digestion of the mucin indicates that the spectrum is largely dependent on the carbohydrate moiety of the glycopeptide.

## Acknowledgments

We are grateful to Dr. William Lynn for providing the bronchial secretion. We also thank Richard Engle for help with the equilibrium ultracentrifugation analysis and Mordecai Moore for the amino acid analysis.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 254, No. 10, Issue of May 25, pp. 4000–4008, 1979
*Printed in U.S.A.*

# The Interaction of Wheat Germ Agglutinin with Sialoglycoproteins

## THE ROLE OF SIALIC ACID*

(Received for publication, September 15, 1978)

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**The role of sialic acid in the interaction of sialoglycoproteins with wheat germ agglutinin was investigated by using several well characterized saccharides and sialoglycoconjugates. *N*-Acetylneuraminic acid and neuramin 2 → 3 lactose, in addition to *N*-acetyl-D-glucosamine and its $\beta 1 \rightarrow 4$ oligomers were found to be inhibitors of wheat germ agglutinin-induced hemagglutination. Neuraminic acid-$\beta$-methyl ketoside, *N*-glycolylneuraminic acid and several other acidic sugars were noninhibitors. Both glycophorin and $\alpha_1$-acid glycoprotein were excellent inhibitors, whereas the corresponding asialo compounds were either very poor or noninhibitors. Among the mucins tested, all except porcine submaxillary mucin, which has *N*-glycolylneuraminic acid, were potent inhibitors. The results of double diffusion experiments, in which the ability of the glycoconjugates to form precipitin line with wheat germ agglutinin was tested, were in agreement with the agglutination inhibition data.**

**Affinity chromatography of labeled glycoconjugates on wheat germ agglutinin-Sepharose 4B columns confirmed that *N*-acetylneuraminyl residues play an important role in the binding of sialoglycoconjugates to this lectin. The results suggest that the binding of a glycoprotein to a wheat germ agglutinin-Sepharose 4B column may be influenced both by the density of the binding sugar residues (*N*-acetyl-D-glucosamine and *N*-acetylneuraminic acid) on the glycoprotein and by the density of the lectin molecules on the gel beads. In cell affinity chromatography, the cells interacted irreversibly with wheat germ agglutinin-Sepharose 6MB beads; this binding was partially lost on treatment of the cells with neuraminidase. Models for the interaction of wheat germ agglutinin with *N*-acetylneuraminic acid and polysialoglycoconjugates are presented.**

**Wheat germ agglutinin is also able to interact with glycoproteins containing multiple nonreducing terminals of *N*-acetylgalactosamine as are present, for example, in asialo ovine and bovine submaxillary mucins.**

In recent years, wheat germ agglutinin (WGA) has been used extensively as a tool for the isolation and characterization of a variety of glycoconjugates, including cell surface components (1–4). Further, the cytotoxic properties of WGA[1] have been utilized for selecting cells with altered cell surface oligosaccharide units (5, 6). In other studies, immunization of mice against the WGA receptor has been tried in an attempt to elicit an immune response in mice against possible wheat germ agglutinin specific tumor antigens (7). Even though a large number of such studies have been carried out with WGA, the true specificity of this lectin remains unclear.

Wheat germ agglutinin, a pure protein with a molecular weight of 36,000, is composed of two similar polypeptide chains, each possessing two binding sites for sugars (8, 9). The binding site consists of three subsites in an extended arrangement rather than in a single deep pocket in the protein (10). WGA is a very stable protein and its stability has been attributed to the presence of a large number of disulfide bridges (8). Several papers have been published on the sugar binding specificity of this lectin (9). It is generally accepted that this lectin binds specifically to 2-acetamido-2-deoxy-D-glucose (GlcNAc) and its $\beta$-1,4-linked oligomers (8, 10–12). In addition, recent studies have provided either direct or indirect evidence for the interaction of sialic acid with WGA (13–20). However, there is no agreement regarding the nature of the interaction between sialic acid or sialyl glycoconjugates and WGA (9, 21). Since WGA is a basic protein, three out of four isolectins having an isoelectric point of 8.7 ± 0.3 (22), the possibility exists of electrostatic interaction between sialic acid and WGA.

While investigating the glycoconjugates produced by murine melanoma cells, we isolated a sialoglycopeptide devoid of GlcNAc but capable of specifically interacting with WGA. It was shown that the interaction of this glycopeptide with WGA was due to the presence of clustered sialyl oligosaccharide groups on the peptide backbone (20). In order to obtain a better understanding as to the real specificity of WGA, a systematic study was undertaken using a large number of simple and complex model saccharides. The results of these investigations are presented in this paper. A preliminary report has been presented (23).

### EXPERIMENTAL PROCEDURES

Raw wheat germ was obtained from local health food stores and from Sigma Chemical Co. Crude wheat germ lectin (B grade) was purchased from Calbiochem.

Ovalbumin (grade III), ovomucoid (trypsin inhibitor), bovine serum albumin, ceruloplasmin (type III), heparin (grade I), trypsin (type I), *N*-acetylneuraminic acid (type VI), *N*-glycolylneuraminic acid, and *p*-nitrophenyl-*N*-acetyl-$\beta$-D-galactosaminide were purchased from Sigma Chemical Co. Chitin, *Vibrio cholerae* neuraminidase, pronase-CB and *p*-nitrophenyl-*N*-acetyl-$\beta$-D-glucosaminide were from Calbiochem. Neuraminic acid-$\beta$-methyl ketoside (methoxyneuraminic acid) and bovine submaxillary mucin were obtained from Boehringer Mannheim. Phenyl-*N*-acetyl-$\alpha$-D-galactosaminide was from Nakarai

* This investigation was supported by Research Grants CA 17686 and CA 15483 from the National Cancer Institute, United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: WGA, wheat germ agglutinin; NaCl/$P_i$, phosphate-buffered saline (0.14 M $Na^+$, 0.1 M $K^+$, 0.006 M $PO_4^{-3}$, pH 7.2); GlcNAc, *N*-acetylglucosamine; NeuNAc, *N*-acetylneuraminic acid; GalNAc, *N*-acetylgalactosamine; NeuNGc, *N*-glycolylneuraminic acid.

Chemicals, Japan. Ribonuclease B, and hyaluronic acid (vitreous humor) were obtained from Worthington Biochemical. Fetuin (Spiro method) and colominic acid were obtained from GIBCO and P-L Biochemicals, respectively. Sugars were mostly from Pfanstiehl. GlcNAc used for elution of WGA-Sepharose columns was prepared by *N*-acetylation (24) of $GlcNH_2 \cdot HCl$ purchased from Pfanstiehl. Sepharose 4B, AH-Sepharose 4B, and cyanogen bromide-activated Sepharose 6MB were purchased from Pharmacia. All other chemicals were commercial products of the highest purity available. [1-$^{14}$C]-Acetic anhydride (4.9 mCi/mmol), [$^3$H]acetic anhydride (50 Ci/mmol), $NaB[^3H]_4$ (330 mCi/mmol) and H[$^{14}$C]HO (45 mCi/mmol) were purchased from New England Nuclear.

Per-*N*-acetylated chitobiose and chitotriose were isolated from chitin by the method of Rupley (25) except that the fractions from the charcoal-Celite column were further purified by chromatography on a Bio-Gel P2 column (26). NeuNAc $\alpha 2 \rightarrow 3$ lactose and NeuNAc $\alpha 2 \rightarrow 3$ [$^3$H]lactitol were prepared as described previously (27). Ovine submaxillary mucin was isolated as described (28). Human bronchial mucin was prepared from bronchial secretions (29). The following were kindly donated as indicated: $\alpha_1$-acid glycoprotein, (The American National Red Cross, Washington, D. C., and from Dr. K. Schmid, Boston University School of Medicine, Boston, Mass.); ovine submaxillary mucin (Dr. M. Horowitz, New York Medical College, Valhalla, N.Y.); bovine submaxillary mucin (Ms. P. Feldhoff, The Milton S. Hershey Medical Center, Hershey, Pa.); porcine submaxillary mucin (Dr. M. de Salequi, Mt. Sinai School of Medicine, New York, N.Y.); heparan sulfate and keratan sulfate II (Dr. M. B. Matthews, University of Chicago, Chicago, Ill.). Gal$\beta$1 $\rightarrow$ 6GlcNAc$\beta$ $\rightarrow$ $C_6H_4NO_2(p)$, Gal$\beta$1 $\rightarrow$ 3GlcNAc$\beta$ $\rightarrow$ $C_6H_4NO_2(p)$, and GlcNAc$\beta$1 $\rightarrow$ 6Gal$\beta$ $\rightarrow$ $C_6H_4NO_2(p)$ (Dr. K. L. Matta, Roswell Park Memorial Institute, Buffalo, N.Y.); di- and trisialogangliosides (Dr. C.-L. Schengrund, The Milton S. Hershey Medical Center, Hershey, Pa.); dog tracheal mucin (30) (Drs. R. Carubelli and G. Sachdev, Oklahoma Medical Research Foundation, Oklahoma City, Okla.); monkey cervical mucin (31) (Dr. Nasir-ud-din, Massachusetts General Hospital, Boston, Mass.); *N*-tosylglucosamine (Dr. M. Miljkovic, The Milton S. Hershey Medical Center, Hershey, Pa.); Jack bean $\beta$-galactosidase (Dr. Y.-T. Li, Tulane University, New Orleans, La.); and *Diplococcus pneumoniae* $\beta$-galactosidase (Dr. J. Umemoto, The Milton S. Hershey Medical Center, Hershey, Pa.).

### *Isolation and Purification of Glycophorin*

Erythrocytes obtained from the blood bank within 1 week of outdating were pooled according to blood group types and the ghosts prepared as described (32). The lyophilized erythrocyte ghosts were treated with lithium diiodosalicylate and the solubilized glycoproteins isolated and washed with ethanol and chloroform:methanol (2:1) as described (33). The major component (glycophorin) was purified by gel filtration on Sepharose 6B (34) followed by chromatography on DEAE-Sepharose 6B using a NaCl gradient (0 to 1 M).

### *Glycophorin Glycopeptides*

Glycophorin and [*NeuNAc*-$^3$H]glycophorin[2] were digested with pronase or TosPhe$CH_2$Cl-trypsin and the glycopeptides from all four digests were purified by chromatography on columns of DEAE-Sephacel using a gradient (0.01 to 1.0 N) of pyridine/acetate. Two major glycopeptide fractions were obtained from each of the pronase and trypsin digests.

The porcine submaxillary mucin glycopeptides and the fetuin glycopeptide Fractions A, B, and C were isolated as described (35). The alkaline borohydride-resistant portion and the tetra- and trisaccharides from fetuin were isolated also as described previously (20, 36). Asialoglycoproteins were prepared either by acid hydrolysis (0.1 N $H_2SO_4$, 80°C, 1 h) or by *Vibrio cholerae* neuraminidase digestion at 37°C for 24 to 48 h in 0.1 M sodium acetate buffer, pH 5.6, containing 0.001 M $CaCl_2$ using 1.0 to 5.0 units of enzyme in a total volume of 200 $\mu$l.

### *Labeling of Glycoproteins and Glycopeptides*[2]

*On Sialic Acid*—The sialic acid residues of sialoglycoproteins and sialoglycopeptides were modified to the 7- and 8-carbon analogs and labeled as described by Van Lenten and Ashwell (37).

[2] [*NeuNAc*-$^3$H], ([$^3$H]galactose), [$^3$H]- or [$^{14}$C]acetyl and [$^{14}$C]dimethyl glycoproteins refers to labeling in sialic acid, galactose, protein by acetylation and protein by reductive methylation, respectively.

*On Terminal Galactose*—Asialoglycoproteins and asialoglycopeptides were labeled on the terminal galactose residue by treatment with galactose oxidase and $NaB[^3H]_4$ (38). Acid hydrolysis followed by paper chromatography showed, as expected, that a major portion of the label was in galactose.

*On Protein*—Radioactive label on the protein was introduced either by acetylation with [$^{14}$C]- or [$^3$H]acetic anhydride or by reductive methylation using H[$^{14}$C]HO and sodium cyanoborohydride (39, 40).

On hydrolysis (6 N HCl, 110°C, 18 h) of the reductively methylated samples followed by analysis on the amino acid analyzer by stream-splitting, about 85% of the label eluted with lysine. In the buffer system used monomethyllysine and dimethyllysine do not separate from lysine (39).

The labeled high molecular weight components were isolated after extensive dialysis, to remove low molecular weight contaminants, followed by lyophilization. The labeled low molecular weight glycopeptides were purified by gel filtration on columns packed with the appropriate supports, generally Sephadex G-25.

### *Isolation of Wheat Germ Agglutinin*

Wheat germ agglutinin was isolated from crude wheat germ by a combination of methods described by Nagata *et al.* (41) and Marchesi (42) with slight modifications. Essentially, the method consisted of extracting the crude wheat germ (200 g) with light petrol (b.p., 38–50°C) twice, using 1 liter each time for 24 h. The air-dried residue was then extracted with 0.05 N HCl, fractionated with ammonium sulfate, and butanol-treated (41). The lectin was purified by affinity chromatography on an ovomucoid or ovalbumin-Sepharose 4B column (42). To understand the process of elution of the lectin from the ovomucoid-Sepharose 4B column, we tested 0.1 M sodium acetate and 10 mM mercaptoethanol in phosphate buffer, pH 7.0, both of which failed to elute the lectin even though small amounts of inactive protein were eluted. Purified WGA purchased from Boehringer Mannheim was also used in our studies.

### *Conjugation of Lectins and Glycoproteins to Sepharose 4B*

Wheat germ agglutinin, ovalbumin, and ovomucoid were coupled to Sepharose 4B which was activated by cyanogen bromide by one of two methods (43, 44). WGA was reacted with the activated Sepharose in the presence of 0.1 M *N*-acetylglucosamine. The remaining active groups were blocked and the gels were washed as described previously (20). The amount of protein bound to Sepharose was determined by estimating the unbound protein in the supernatant and washes. WGA-Sepharose 6MB purchased from Pharmacia was also used in some of our experiments.

### *Affinity Chromatography of Glycoproteins on WGA-Sepharose 4B Columns*

The sample, usually radioactively labeled, was applied in a volume of 100 to 500 $\mu$l to a lectin column (2 to 5 ml of gel) equilibrated with the elution buffer (50 mM Tris-HCl, pH 8.0, with or without 0.1% Triton X-100). The sample was washed twice into the column with about 0.5 ml of buffer and allowed to incubate for about 15 min. The column was then eluted with the above buffer followed by 0.1 M *N*-acetylglucosamine in the same buffer and fractions of 1 ml were collected. The flow rate of the column was controlled to about 10 to 15 ml/h. The radioactivity in the eluted fractions was measured after mixing with 10 ml of scintillation counting liquid. The total radioactivity recovered from the columns was better than 85% unless otherwise mentioned. The columns were routinely regenerated by washing with a minimum of 10 bed volumes of the starting buffer. Occasionally the columns were cleaned, particularly when the recoveries have been poor, by washing the column with 6 M urea, followed by buffer containing 0.1% Triton X-100 and finally the starting buffer.

The columns were stored between experiments at 4°C in 0.1 M *N*-acetylglucosamine in buffer containing 0.02% sodium azide; most of our WGA-Sepharose 4B preparations have remained active over periods of 6 months to 1 year.

Other buffers, including those containing between 0.1 to 0.5 M NaCl, were also tried but had no significant effect on the results (45).

### *Agglutination and Agglutination-Inhibition Assays*

Human blood group (O)H erythrocytes were washed and a 2% suspension of cells in $Ca^{2+}$- and $Mg^{2+}$-free NaCl/$P_i$ was prepared. For the titration of lectin activity, serial 2-fold dilutions of the stock lectin

solutions were prepared in siliconized microtest slides (A. H. Thomas, Philadelphia) in a volume of 50 μl of NaCl/$P_i$. An equal volume of the erythrocyte suspension was added to each well and the slide was incubated on a shaker at room temperature for 20 min. The degree of hemagglutination was scored on a serological scale from 0 (no agglutination) to +4 (maximum agglutination).

For agglutination inhibition assays, the inhibitors dissolved in NaCl/$P_i$ and pH adjusted to 7.0 if necessary, were serially diluted in the plates in a volume of 40 μl. The minimum amount of WGA (for untreated erythrocytes, 1 μg in 10 μl of NaCl/$P_i$ giving a final concentration of 10 μg/ml) required to give complete agglutination in the absence of inhibitor was then added to each well and mixed, and 50 μl of the erythrocyte suspension was added and once again mixed. After shaking the mixture on a rotary shaker at room temperature for 20 min, the plates were examined under a microscope and the degree of agglutination in each well was assessed to determine the concentration of the inhibitor causing 50% inhibition. As pointed out by Allen *et al.* (10), such an assay involving visual scoring is only semiquantitative and is liable to give an error of ±20%.

### *Precipitation Analysis by Diffusion in Gel*

Double diffusion was carried out in immunodiffusion plates (Miles) using 2% agar in 0.15 M phosphate buffer, pH 7.2 (46). The center well was filled with 20 μl of a solution of WGA, 2 mg/ml in NaCl/$P_i$ and outer wells were filled with glycoprotein solutions at 2 mg/ml in water or NaCl/$P_i$; certain glycoproteins were also tested at higher concentrations. The diffusion plates were incubated in a humid chamber for 18 to 48 h. Some plates were washed, dried, and stained with Amido black prior to visualization (47).

## RESULTS

### *Further Purification of WGA*

Attempts to purify further the lectin by a second chromatography on the same affinity column always resulted in extremely poor recovery. In a typical experiment, 186 mg of WGA was applied to an ovomucoid-Sepharose 4B column; elution with 0.1 N HOAc yielded only 25 mg of protein which had the same hemagglutinating activity as the original lectin. Elution of the column with 1 M acetic acid, 0.05 N HCl, or 0.1 M GlcNAc did not yield any additional protein. In another experiment, 200 mg of a crude wheat germ lectin preparation (Calbiochem, B grade, Lot 430031) having an activity equivalent to at least 50 mg of pure lectin yielded 3 mg of active protein after affinity chromatography using an ovomucoid column. Similar results were also obtained when repurification was attempted on a column of Sepharose substituted with *p*-aminophenyl-2-acetamido-2-deoxy-D-glucopyranoside[3] (48).

### *Hemagglutination-Inhibition Assays*

The results of the hemagglutination-inhibition experiments are summarized in Tables I to III. The results reported were reproducible in several experiments. There were no significant differences in the inhibitory effect of the saccharides using either our WGA preparations or those obtained from Boehringer Mannheim. The glycoconjugate inhibitors listed in Tables II and III did not cause agglutination or lysis of human group O erythrocytes in the concentration range used in our experiments. Our results on hexosamines (Table I) are in agreement with published results that *N*-acetylglucosamine and its derivatives are in general good inhibitors, whereas *N*-acetylgalactosamine and *N*-acetylmannosamine are poor inhibitors (8, 10). The *p*-nitrophenyl glycosides of GlcNAc and its oligosaccharides are all better inhibitors than GlcNAc. Of major interest to this study was the inhibitory activity of neuraminlactose and NeuNAc, the former being a better inhibitor than GlcNAc, whereas lactose was noninhibitory up to 200 mM. It is not clear why neuraminlactose is a more potent inhibitor than NeuNAc. The effect was specific for NeuNAc, since NeuNGc, neuraminic acid-β-methyl ketoside and other acidic sugars, such as muramic, glucuronic, and glucosaminic acids, were noninhibitory. Of the glycoproteins with mainly *N*-glycosidically linked carbohydrates (Table II), the inhibitory activity of ovomucoid, ovalbumin, and ribonuclease B is due to GlcNAc residues in these molecules. On the other hand, the inhibition by $\alpha_1$-acid glycoprotein is probably due to the terminal NeuNAc residues since the asialo compound was not inhibitory at 4 times the concentration. Fetuin was a weak inhibitor compared to $\alpha_1$-acid glycoprotein and ovomucoid. Various derivatives of fetuin including asialo fetuin were all noninhibitors. The weak inhibition by fetuin was surprising in view of the fact that all other results indicate an efficient interaction between fetuin and WGA. For example, WGA could be isolated using a column of fetuin-Sepharose 4B[4] (50), fetuin gave a precipitin line with WGA and labeled fetuin bound to affinity columns of WGA-Sepharose 4B (Table IV).

TABLE I

*Inhibitory effect of simple and complex saccharides on the agglutinating activity of wheat germ agglutinin*

| Compounds | Concentration needed for 50% inhibition |
|---|---|
| | *mM* |
| GlcNAc | 12.5 |
| *N*-Tosylglucosamine | >40[a] |
| GlcNAcβ1 → 4GlcNAc | 0.15 |
| GlcNAcβ1 → 4GlcNAcβ1 → 4GlcNAc | 0.03 |
| GlcNAcβ → $C_6H_4NO_2(p)$ | 0.10 |
| Galβ1 → 6 GlcNAcβ → $C_6H_4NO_2(p)$ | 0.125 |
| Galβ1 → 3 GlcNAcβ → $C_6H_4NO_2(p)$ | 2.0 |
| GlcNAcβ1 → 6Galβ → $C_6H_4NO_2(p)$ | 0.125 |
| ManNAc | 200 |
| GalNAc | 200 |
| GalNAcα → phenyl | >4.0[a] |
| GalNAcβ → $C_6H_4NO_2(p)$ | >3.2[a] |
| NeuNAc α2 → 3 lactose | 6.25 |
| NeuNAc | 25 |
| Neuraminic acid β-methyl ketoside | >80 |
| NeuNGc | >200 |
| Lactose | >200 |
| Muramic acid | >160 |
| Glucosaminic acid | >125 |
| Tryptophan | >200 |
| Glucuronic acid | >332 |

[a] Higher concentrations could not be tested due to poor solubility.

TABLE II

*Inhibitory effect of glycoconjugates on the agglutinating activity of wheat germ agglutinin*

| Compounds | Concentration needed for 50% inhibition | |
|---|---|---|
| | *μM* | *μg/ml* |
| $\alpha_1$-Acid glycoprotein[a] | 1.4; 4.5 | 62.5; 200 |
| Asialo $\alpha_1$-acid glycoprotein | >20.8 | >800 |
| Fetuin | 20.8 | 1000 |
| Asialo fetuin | >52.7 | >2160 |
| Fetuin glycopeptides A | >200 | >800 |
| Fetuin glycopeptides B and C | | >400 |
| Alkaline borohydride-resistant fetuin | >88.9 | >4000 |
| Ovomucoid | 2.2 | 62.5 |
| Ovalbumin | 4.2 | 187.5 |
| Ribonuclease B (Worthington) | >54.4 | >800 |
| Ribonuclease B (purified)[b] | 4.25 | 62.5 |

[a] Two different preparations, one from Dr. Karl Schmid and the other from the American Red Cross, gave two different values.

[b] The fraction bound on concanavalin A-Sepharose 6B column and eluted with α-methylmannoside.

[3] J. R. Banks, V. P. Bhavanandan, and E. A. Davidson, unpublished results.

[4] V. P. Bhavanandan and A. Katlic, unpublished results.

The most powerful inhibitors of WGA agglutinations are mucins or glycophorin, which has a mucin-type structure having several clustered sialyl oligosaccharides linked *O*-glycosidically to the peptide backbone (51) (Table III). Ovine submaxillary mucin and glycophorin were 50,000 and 70,000 times more potent than GlcNAc in inhibiting WGA. Of the mucins tested, the only one which did not show inhibition was pig submaxillary mucin. This is interesting since about 90% of the sialic acid in this mucin is *N*-glycolyl (52). Glycopeptides isolated from glycophorin were also inhibitory, but it is not possible to compare their potency against glycophorin and GlcNAc because of the lack of information on their molecular weights. The noninhibitory nature of polyanions such as hyaluronic acid, heparan sulfate, and keratan sulfate (all GlcNAc-containing) confirms the specific nature of the inhibition by NeuNAc-containing polyanionic mucins. The nonacidic desulfated keratan sulfate has been shown to form a precipitate with WGA (53). This is attributable to the alternating 1 → 4-linked GlcNAc in this polysaccharide; desulfation would also remove the NeuNAc from this polymer (54). A sialic acid polymer, colominic acid, was also noninhibitory. Colominic acid is a linear polysaccharide of sialic acid linked $\alpha 2 \rightarrow 8$; it is possible that such a polymer would be unable to interact effectively with sugar binding subsites of WGA (see "Discussion"). A mixture of di- and trisialogangliosides did not inhibit WGA-induced hemagglutination.

TABLE III

*Inhibitory effect of glycoconjugates on the agglutinating activity of wheat germ agglutinin*

| Compounds | Concentration needed for 50% inhibition | |
|---|---|---|
| | μM | μg/ml |
| Glycophorin (MM)[a] | 0.16 | 5 |
| Glycophorin (NN)[a] | 0.32 | 10 |
| Glycophorin glycopeptides (trypsin) | | 100 |
| Glycophorin glycopeptides (pronase) | | 200 |
| Asialoglycophorin | 16.3; >32.5 | 400[b]; >800[c] |
| Ovine submaxillary mucin[d] | 0.23 | 35 |
| Bovine submaxillary mucin[e] | 0.32–0.65[f] | 50–100 |
| Porcine submaxillary mucin | >0.52[f] | >800 |
| Porcine submaxillary mucin glycopeptides | | >800 |
| Asialo ovine submaxillary mucin | 1.85; 0.23 | 200[b]; 25[c] |
| Asialo bovine submaxillary mucin | 0.32; 0.92[f] | 50[b]; 100[c] |
| Human bronchial mucin | | 25 |
| Dog tracheal mucin | | 50 |
| Monkey cervical mucin | | 30 |
| Hyaluronic acid | | >2000 |
| Heparan sulfate | | >2000 |
| Keratan sulfate II | | >2000 |
| Colominic acid | | >800 |
| Di- and trisialogangliosides | | >1000 |

[a] MM and NN designate human blood group type O(MM) and O(NN).

[b] Asialo compound prepared by acid hydrolysis.

[c] Asialo compound prepared by treatment with *V. cholerae* neuraminidase.

[d] Our preparation; the sample donated by Dr. Horowitz gave comparable results.

[e] Preparation gifted by Ms. Feldhoff. Preparation from Boehringer Mannheim gave a value of 50 μg/ml.

[f] These values are approximate since the exact molecular weight of these mucins are not known. A value of 154,000 reported (49) for ovine submaxillary mucin was used for these mucins as well.

Asialo ovine submaxillary mucin and asialo bovine submaxillary mucin also inhibited agglutination by WGA, although at a slightly higher concentration than the native mucins (see also Ref. 21). These results would tend to suggest that NeuNAc has no role in the inhibition of WGA agglutination by these mucins. On the other hand, it is likely that the large numbers of nonreducing terminal GalNAc in these asialo mucins are responsible for inhibition. It should be pointed out that GalNAc is an inhibitor, even though only at higher concentrations (200 mM) compared to NeuNAc (25 mM) and GlcNAc (12.5 mM). Thus, a peptide with multiple, some clustered, GalNAc would be expected to be a good inhibitor. In order to confirm the role of GalNAc in the asialomucin in the interaction with WGA, in preliminary experiments,[4] we tested bovine submaxillary mucin and ovine submaxillary mucin which were deglycosylated by treatment with hydrogen fluoride (55). It was found that these preparations free of sialic acid and about 90% of the GalNAc did not inhibit WGA agglutination up to about 7 μM. Higher concentrations could not be tested due to the poor solubility of the deglycosylated samples.

Asialoglycophorin was a poor inhibitor compared to the asialo mucins. This is consistent with the fact that the nonreducing terminals in this compound are galactose. The weak inhibition by this compound could be due to the internal GlcNAc residues present in the *N*-glycosidically linked prosthetic group, even though the possibility of contribution from the terminal galactose residues to the interaction cannot be completely discounted (19). Interaction between the internal GlcNAc in carcinoembryonic antigen and WGA was suggested by Goldstein *et al.* (12).

### *Double Diffusion Experiments*

The results of a few typical double diffusion experiments using wheat germ agglutinin and various glycoproteins are shown in Fig. 1.

TABLE IV

*Interaction of labeled glycoproteins with different preparations of WGA-Sepharose 4B*

All glycoproteins were labeled on the protein by acetylation; sialoglycoproteins were also labeled on sialic acid residues.

| WGA-Sepharose 4B preparation number[a] | Per cent radioactivity specifically bound | | | | | | |
|---|---|---|---|---|---|---|---|
| | Glycophorin | Asialoglycophorin | Fetuin | Asialo fetuin | Ovomucoid | $\alpha_1$-Acid glycoprotein | Pig submaxillary mucin |
| 1 | 94 | N.T.[b] | 30[c]; 33; 35 | N.T. | 16; 30; 21 | 11 | 9 |
| 2 | 95 | 47 | 63; 66[d] 73[c]; 80 | 1.5 | 40; 46 | 52 | N.T. |
| 3 | 99; 100 | 37 | 90; 93 88; 96 | 0 | 96 | 100 | 34 |

[a] WGA isolated by affinity chromatography on immobilized ovomucoid was used in the preparations. Based on the estimations of the protein not bound to Sepharose, the Preparations 1, 2, and 3 should have approximately 1, 2, and 5 mg of bound protein/ml of gel. WGA-Sepharose 6MB purchased from Pharmacia and containing 5 mg/ml of gel gave results comparable to Preparation 3.

[b] N.T., not tested.

[c] Ten times more sample applied.

[d] Fifty times more sample applied.

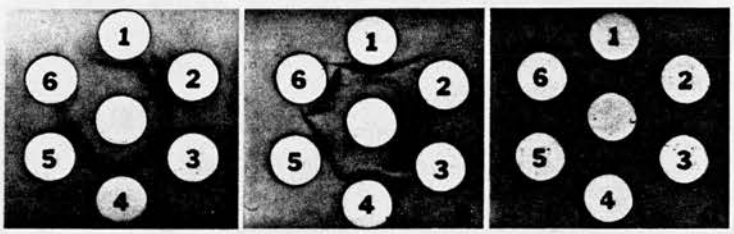


FIG. 1. Double diffusion reaction of WGA in the *center well* against various glycoconjugates in the *outer wells*. *A: 1*, ceruloplasmin; *2*, $\alpha_1$-acid glycoprotein; *3*, fetuin; *4*, ovomucoid; *5*, ovalbumin; *6*, asialofetuin. *B: 1* and *5*, glycophorin MM; *2*, asialoglycophorin MM; *3*, glycophorin glycopeptides (trypsin); *4*, glycophorin NN; *6*, glycophorin glycopeptides (pronase). *C:1*, porcine submaxillary mucin (a weak band was visible soon after development); *2*, di- and trisialogangliosides; *3*, asialo ovine submaxillary mucin; *4*, ovine submaxillary mucin; *5*, asialo bovine submaxillary mucin; *6*, bovine submaxillary mucin. Other details are given under "Experimental Procedures."

At concentrations of 2 mg/ml, ovine submaxillary mucin, bovine submaxillary mucin, human bronchial mucin, dog tracheal mucin, monkey cervical mucin, glycophorin, and glycophorin glycopeptides all showed strong precipitin bands. In the case of glycophorin, a band was detectable even at a concentration of 200 $\mu$g/ml. Glycoconjugates which gave weak bands at 2 mg/ml include porcine submaxillary mucin, asialo submaxillary mucins, and asialoglycophorin. Ovalbumin and fetuin gave strong bands at 15 mg/ml and very weak bands at 5 mg/ml; in comparison, ovomucoid and $\alpha_1$-acid glycoprotein formed strong bands at 5 mg/ml and weak bands at 2 mg/ml.

Several other components failed to produce a precipitin band against WGA at concentrations as high as 15 mg/ml. These include asialo fetuin, bovine serum albumin, hyaluronic acid, chondroitin 4-sulfate, heparin, and di- and trisialogangliosides.

### *Affinity Chromatography of Glycoconjugates on WGA-Sepharose Columns*

Except for those experiments reported in Table IV, all others were carried out using fully active WGA-Sepharose 4B columns, comparable to Preparation 3 in Table IV.

*Glycophorin*—Glycophorin isolated from human erythrocytes of blood group MM or NN and labeled either in sialic acid or in the protein was completely retained on various WGA-Sepharose columns (Table IV and Fig. 2). The results obtained when a typical column was eluted with various solutions prior to elution with 0.1 M GlcNAc is shown in Fig. 3. Asialoglycophorin preparations isolated either by neuraminidase or acid treatment of the glycophorin were only partially (35 to 50%) retained on the WGA columns (Table IV and Fig. 2). All four major glycopeptides isolated from [*NeuNAc*-$^3$H]glycophorin by pronase or trypsin digestion were retained on the WGA column (Fig. 2). When [*NeuNAc*-$^3$H]glycophorin was treated with alkaline borohydride and chromatographed on a Bio-Gel P6 column, a large included peak was obtained eluting in the position of tetrasaccharides isolated from fetuin by similar treatment (36). These tetrasaccharides were not retained on the WGA column.

*Fetuin*—Different proportions of fetuin labeled in the protein either by reductive methylation or acetylation were retained on different WGA-Sepharose 4B columns as can be seen from Table IV. Once again prior elution of the column with salts or acetic acid did not displace a significant portion of the fetuin (Fig. 3). Fetuin glycopeptides A, B, and C labeled by reductive methylation were not bound to the WGA column which completely retained the native fetuin labeled by the same method. The alkaline borohydride-resistant portion of fetuin also was not retained on the affinity column. Asialo

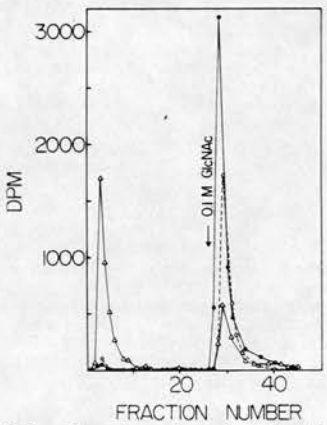


FIG. 2. Affinity chromatography of [$^3$H]acetyl glycophorin (●——●), [$^3$H]acetyl asialoglycophorin (△——△), and a [NeuNAc-$^3$H]glycophorin glycopeptide isolated by pronase treatment of [NeuNAc-$^3$H]glycophorin (○- - -○) on a WGA-Sepharose 4B column. Elution was with 50 mM Tris-HCl, pH 8.0, followed by 0.1 M GlcNAc in buffer. One-milliliter fractions were collected and analyzed for radioactivity. One other glycopeptide isolated by pronase treatment and the two glycopeptides isolated by trypsin treatment of [NeuNAc-$^3$H]glycophorin were also completely retained on this column.

fetuin prepared by neuraminidase treatment was not bound on any of the WGA columns tested (Table IV). This compound, however, was completely bound to and eluted from a *Ricinus communis* Agglutinin I-Sepharose 4B column, indicating the presence of terminal galactose residues. Treatment of the asialo fetuin with either *D. pneumoniae* $\beta$-galactosidase for 2 h or with jack bean $\beta$-galactosidase for either ½ or 6 h abolished its ability to bind to the *Ricinus communis* Agglutinin I column, but the products still were not retained on the WGA column.

*Other Glycoconjugates*—Ovine and bovine submaxillary mucins labeled on sialic acid were completely retained on WGA-Sepharose columns. Ovomucoid labeled in the protein moiety was retained on different WGA columns to different degrees as shown in Table IV. $\alpha_1$-Acid glycoprotein labeled either on sialic acid or protein behaved similarly (Table IV). Asialo $\alpha_1$-acid glycoprotein prepared by neuraminidase treatment of the [$^3$H]acetyl $\alpha_1$-acid glycoprotein was not retained on any of the WGA columns tested. On affinity chromatography of [$^{14}$C]acetyl porcine submaxillary mucin 9 and 34% were retained on two different WGA columns (Table IV).

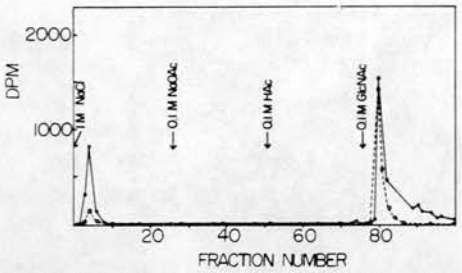


FIG. 3. Chromatography of [$^3$H]acetyl fetuin (○– – –○) and [$^3$H]acetyl glycophorin (●——●) on WGA-Sepharose 4B columns. After application of the sample and a 15-min incubation, the columns were sequentially washed with 50 mM Tris-HCl buffer, pH 8.0, 1 M NaCl in buffer, 0.1 M NaOAc, pH 8.1, 0.1 M acetic acid, and finally 0.1 M GlcNAc in buffer. Fractions of 1 ml were collected and radioactivity was determined.

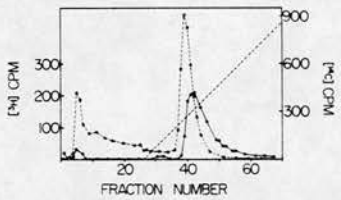


FIG. 4. Affinity chromatography of [$^{14}$C]acetyl fetuin (○– – –○) and [$^3$H]acetyl glycophorin (●——●) on a WGA-Sepharose 4B column. The mixed samples were applied on the column and eluted with 50 mM Tris-HCl, pH 8.0, followed by a linear gradient (– – –) of buffer alone (10 ml) to 0.1 M GlcNAc in buffer (10 ml). Fractions of 1 ml (1 to 25) and 0.5 ml (26 to 65) were collected and analyzed for radioactivity.

Chondroitin sulfate and heparan sulfate, isolated from human melanoma HM$_7$ cells grown in the presence of [$^3$H]glucosamine,[4] were not retained on the WGA columns. NeuNAc 2 → 3 [$^3$H]lactitol was also not retained on the WGA column. [$^{14}$C]Dimethyl ribonuclease B, purified on concanavalin A-Sepharose 4B column, was partially (35%) retained on the WGA column. [$^3$H]Acetyl ovalbumin glycopeptides were not bound to this column.

### *Elution of the WGA-Sepharose 4B Columns with GlcNAc Gradient*

Since the various glycoproteins appeared to interact differently with the immobilized WGA, the possibility of separating these glycoproteins by affinity chromatography using a gradient (0 to 100 mM) of GlcNAc was tested. Fetuin and $\alpha_1$-acid glycoprotein appeared to elute at the same position (45 mM GlcNAc) in the gradient. Glycophorin, however, appeared to elute a few fractions after fetuin and $\alpha_1$-acid glycoprotein. The result of a mixed experiment using [$^{14}$C]acetyl fetuin and [$^3$H]acetyl glycophorin is shown in Fig. 4.

### *Cell Affinity Chromatography*

In an attempt to find out whether cultured cells could be fractionated, cell affinity chromatography was carried out using a K9/15 column with 80-μm nylon net (Pharmacia) packed with 2 ml of WGA-Sepharose 6MB gel. Human breast cells (HBL-100 and MDA-321) (56) grown in the presence of [$^3$H]thymidine were harvested, washed three times with NaCl/P$_i$ and fixed with formaldehyde (10% HCHO in NaCl/P$_i$ 1 h at room temperature). A suspension of the cells in NaCl/P$_i$ was applied to the column, and the column was washed with NaCl/P$_i$ and eluted with 0.1 M GlcNAc. The recoveries were extremely low as can be seen in Table V. The cells recovered from the column increased more than 5-fold when the fixed MDA-231 cells were treated with *V. cholerae* neuraminidase (37°C, 24 h) prior to affinity chromatography. This suggested that the binding of the cells to the WGA-Sepharose gels was probably mediated by cell surface sialyl residues. On applying formaldehyde fixed MDA-321 cells to the column which was pre-equilibrated with 0.1 M GlcNAc, 52% of the radioactivity was not retained. Similar results were obtained with unfixed cells.

TABLE V

*Chromatography of [$^3$H]thymidine-labeled cells on columns of WGA-Sepharose 6MB*

| Cells | Radioactivity | | |
|---|---|---|---|
| | Applied | Washed with buffer | Eluted with 0.1 M GlcNAc |
| | | *dpm* | |
| MDA-231 cells (untreated) | 50,000 | 3,050 (6.1%) | 2,500 (5.0%) |
| HBL-100 cells (untreated) | 57,000 | 2,533 (4.4%) | 716 (1.3%) |
| MDA-231 cells treated with neuraminidase | 20,065 | 10,377 (51.7%) | 2,175 (10.8%) |
| MDA-231 cells mixed and applied in 0.1 M GlcNAc | 20,758 | | 10,883 (52.4%) |

## DISCUSSION

Wheat germ agglutinin was isolated by affinity chromatography on ovomucoid (or ovalbumin) Sepharose 4B columns. The yields of lectin varied from batch to batch of crude wheat germ. Usually between 50 to 200 mg of the lectin/200 g of wheat germ were obtained; however, certain batches of crude wheat germ gave extremely poor yields (<10 mg). The length of storage of the crude wheat germ prior to use may be one of the reasons for this variation in yield since one batch of raw wheat germ which initially gave about 100 mg of lectin/200 g after storage for about a year at 4°C yielded only 7 mg. In addition to this, it is not clear whether the strain of the wheat, condition, and length of storage of wheat before isolation of the germ, and other unknown factors influence the yield. A most puzzling aspect of WGA isolation was the loss of material incurred during attempted repurification. On rechromatography of the isolated lectin on affinity columns, the protein apparently bound to the ligand irreversibly and could not be displaced. This aspect was not further investigated.

WGA has been purified by affinity chromatography on immobilized ovomucoid, fetuin, synthetic GlcNAc-based adsorbents or on chitin (9, 50). We found that immobilized bovine submaxillary mucin (which lacks GlcNAc but contains NeuNAc) was also effective for the isolation of WGA.[4] In control experiments,[4] WGA did not bind to columns packed with immobilized bovine serum albumin confirming the specificity of the interaction between WGA and GlcNAc or NeuNAc-containing ligands, or both.

The inability of sodium acetate to elute the WGA from affinity columns indicates that even though *N*-acetyl groups of the hapten sugars (GlcNAc, NeuNAc, and GalNAc) are important for the interaction, acetate ions are not capable of disrupting the complex between WGA and the ligand. Mercaptoethanol, which was expected to reduce the large number of —S—S— bonds in WGA, also failed to disrupt the inter-

action between the lectin and the ligand under the conditions used.

The hemagglutination inhibition experiments, while confirming earlier reports that GlcNAc-containing compounds are good inhibitors of WGA-induced hemagglutination, provide clear evidence for the inhibitory ability of NeuNAc-containing compounds. Nonspecific interaction between NeuNAc and WGA could be excluded by the inability of several other acidic sugars to inhibit agglutination. The importance of the *N*-acetyl group in NeuNAc (and NeuNAc 2 → 3 lactose) is shown by the lack of inhibition by neuraminic acid $\beta$-methyl ketoside with a free $NH_2$ group and by NeuNGc.

In double diffusion experiments, the macromolecular glycoconjugates which were good inhibitors of hemagglutination were also the ones which gave precipitation lines with WGA.

Previous studies with sialoglycopeptides isolated from mouse and human melanoma cells (20, 57) indicated that glycopeptides having clustered *O*-glycosidically linked sialyl oligosaccharides were capable of interacting specifically with WGA. It was also apparent that the interaction was through sialic acid residues since the removal of sialic acid abolished the interaction. The major sialoglycoprotein of the human erythrocyte membrane is known to have 15 *O*-glycosidically linked sialyl tetrasaccharides in addition to 1 *N*-glycosidically linked oligosaccharide (51). Fetuin has three each of *N*-glycosidically and *O*-glycosidically bound oligosaccharides (36). Therefore, in order to confirm our previous findings, we chose these and other well characterized glycoproteins for further studies using affinity chromatography. The results obtained with fetuin, glycophorin, and $\alpha_1$-acid glycoprotein confirm the role of NeuNAc in the binding of these components to WGA. Asialo fetuin and asialo $\alpha_1$-acid glycoprotein were not retained on the lectin column; thus, the terminal sialic acids are the sites of interactions. The specificity of the binding is illustrated by the inability of sodium chloride, acetic acid, or sodium acetate to displace the bound fetuin or glycophorin (Fig. 3).

The necessity for the presence of several sialyl oligosaccharides on a peptide backbone for stable binding is shown by the failure of neuraminyl lactitol, sialyl tetrasaccharide (isolated by alkaline borohydride treatment of glycophorin or fetuin), and fetuin glycopeptide Fractions A, B, and C to bind to the affinity column. It should be noted that, whereas neuraminyl lactitol and sialyl tetrasaccharide have 1 and 2 NeuNAc residues, respectively, fetuin glycopeptide Fraction A could have up to 3 terminal NeuNAc residues and 5 internal GlcNAc residues (58). In contrast, the glycopeptides isolated from glycophorin all bound to the WGA column in agreement with the fact that these glycopeptides would have multiple sialyl oligosaccharides (51). These results indicate clearly the requirement of the macromolecular structure and the polyvalent binding sites of the glycoconjugates for their stable binding to WGA-Sepharose column. Further work is necessary before the minimum valency required for stable interaction can be defined.

The role of internal GlcNAc residues in the interaction between glycoconjugates and WGA columns is not clear since asialo fetuin and asialo $\alpha_1$-acid glycoprotein were not retained on WGA columns but asialoglycophorin was partially retained. The failure of asialo agalactofetuin having nonreducing termini of GlcNAc and GalNAc to be retained on WGA columns was unexpected and difficult to understand. One possible explanation for this finding is that the spacing of GlcNAc and GalNAc terminals on the protein is unsuitable for stable binding to the WGA immobilized on Sepharose beads.

In agreement with the results from agglutination inhibition experiments, porcine submaxillary mucin was only partially retained on the lectin column probably because of the *N*-glycolylneuraminic acid present on this glycoprotein. The retained portion may be molecules with human blood group A activity and thus having nonreducing GalNAc terminals. Contributions from the NeuNAc residues, 10% of the total sialic acid, to this binding is also possible. The porcine submaxillary mucin used in these studies was from pooled pig submaxillary glands.

The finding that the proportions of fetuin, $\alpha_1$-acid glycoprotein, and ovomucoid binding to different WGA-Sepharose 4B preparations varied, in contrast to glycophorin which bound completely to all the preparations, is difficult to interpret (Table IV). Since applying 10 and 50 times more of the samples to the lectin columns 1 and 2 did not alter the results, it is clear that the partial binding of fetuin and ovomucoid was not due to overloading the columns. The variation in the concentration of the lectin on the gel could be a factor influencing the binding of different proportions of glycoproteins to different preparations. In other words, the interaction between a sialoglycoprotein and immobilized WGA could be influenced by both the density of GlcNAc or NeuNAc residues on the glycoprotein and the density of WGA molecules on the gel bead. Thus, a WGA-Sepharose 4B preparation with high density of lectin on the gel beads is capable of binding several glycoproteins completely, including those with a low density of NeuNAc or GlcNAc on the protein (fetuin, $\alpha_1$-acid glycoprotein, and ovomucoid). In contrast, an affinity gel preparation with a low density of lectin on the gel will bind completely those glycoproteins with a high density of NeuNAc, such as glycophorin, and interact only partially with the other glycoproteins. The proportions of fetuin and ovomucoid retained on these columns apparently reflects the heterogeneity of these molecules with regard to distribution of terminal NeuNAc and GlcNAc available for interaction. Further investigation of this aspect is necessary to test this hypothesis.

The results of this study indicate that the interaction of glycoproteins with WGA is complex. A well recognized feature of this interaction is the role of GlcNAc and its $\beta 1 \rightarrow 4$-linked oligomers in glycoproteins which bind to subsites postulated by Allen and co-workers (10). These workers also reported that, for efficient binding, the equatorial acetamido group at position 2 and the equatorial hydroxy group at position 3 of the hexosamine are essential. In an analogous manner, NeuNAc could interact at the same site via the acetamido group at position 5 and the hydroxy group at position 4 as illustrated in Fig. 5. Since the sialoglycopeptides labeled by modifying the NeuNAc residues to 8- and 7-carbon analogs still interacted with WGA, the side chains involving carbon atoms 7, 8, and 9 in NeuNAc are most probably not involved in the binding and in fact may even hinder efficient binding. In the case of sialoglycoproteins, up to 3 sialyl residues present in a suitable arrangement could interact at the sugar binding regions, thus leading to the formation of an eventually stable complex between the glycoprotein and WGA as illustrated in Fig. 6. It is not clear whether 3 sialic acid residues as presented by the oligosaccharides attached to consecutive amino acids, for example, as in glycophorin, or the terminal sialic acids present in the branched *N*-glycosidically linked oligosaccharides could interact at three consecutive subsites without hindrance. If such an interaction is prevented due to the larger size of the NeuNAc residue, compared to the GlcNAc residue, than a stable complex could still be formed by the interaction of only 1 or 2 NeuNAc residues at each receptor region of WGA. Further studies are needed to obtain additional information as to the exact mechanism of the interaction between sialoglycoconjugates and WGA.

If the above mentioned postulate is correct, it would be

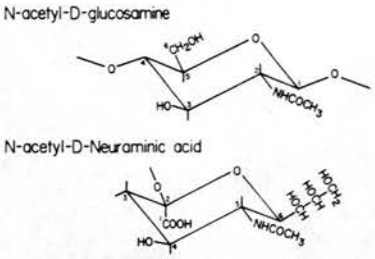


FIG. 5. Structures of NeuNAc and GlcNAc in the 1C and C1 conformations, respectively. The similar (*trans*) relationship between the acetamido (position 5 of NeuNAc and 2 of GlcNAc) and the hydroxyl (position 4 of NeuNAc and 3 of GlcNAc) groups in both structures is evident.

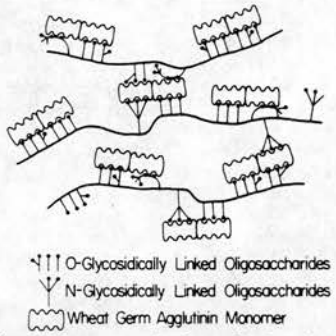


FIG. 6. Illustration representing the multivalent interaction of a glycoprotein with WGA. The WGA molecule is depicted as a dimeric protein with four carbohydrate-binding regions, each capable of interacting with up to 3 NeuNAc or GlcNAc residues. The glycoprotein molecules have oligosaccharides linked both *O*-glycosidically and *N*-glycosidically to the protein. The model is not drawn to scale; only some of the sugar residues are indicated by ● (terminal NeuNAc residues) and by ○ (internal GlcNAc residues in the *N*-glycosidic linkage region interacting with WGA). Maximum interaction is shown, however, this may not be possible always due to stereochemical reasons. For example, interactions of only 1 sugar residue (NeuNAc or GlcNAc)/binding region may be sufficient to lead to a stable complex.

possible to explain how a glycoprotein having only GalNAc, such as asialo ovine submaxillary mucin or asialo bovine submaxillary mucin, could lead to the formation of a stable complex with WGA. It is known that the configuration at position 4 of an *N*-acetylhexosamine is important for the interaction with WGA (10). When the 4-hydroxyl group is equatorial with or without substitution, as in GlcNAc and its 4-substituted derivatives, favorable binding occurs. Replacement by an axial 4-hydroxyl group as in GalNAc leads to weak binding, since GalNAc had 1/16 of the potency of GlcNAc (Table I). Asialo ovine submaxillary mucin having several GalNAc on the protein backbone (on an average one in six amino acids are substituted (52)) will interact through these sugar residues, leading to a favorable stable complex. Thus, it is clear that the specificity of WGA and of course of other lectins is relative rather than absolute. In the case of WGA, at the present at least three sugars (GlcNAc, NeuNAc, and GalNAc) commonly found in glycoconjugates appear capable of interacting with different affinities. Depending on several other factors glycoconjugates containing these sugars could form stable complexes. The situation of WGA binding to the cell surface would thus be more complicated consisting for example of low and high affinity binding sites as suggested earlier (4, 6).

Four forms (isolectins) of WGA having similar properties have been isolated (10, 22). It will be interesting to determine whether these isolectins have different specificities with respect to GlcNAc, NeuNAc, and GalNAc.

In preliminary experiments, we have observed that the recovery of labeled glycoproteins is usually poor from lectin columns with a high (>5 mg/ml) concentration of lectin on the gel (compare the 6% recovery of WGA receptor activity from erythrocytes (4)). This suggests the possibility of strong irreversible interaction between glycoproteins and gels with a very high density of lectin molecules. The multivalent interaction between a glycoprotein and WGA will vary from very weak to very strong, depending on the factors discussed above and the possible cooperativity of the interaction. Positively cooperative binding of $^{125}$I-WGA to fat cells has been reported by Cuatrecasas (14). Thus, a monovalent hapten inhibitor, such as GlcNAc, would not be capable of interrupting a strong interaction between a polyvalent glycoprotein and WGA, thus explaining the poor recovery mentioned above. The results of the cell affinity chromatography are in agreement with this; the very high density of sialyl and or *N*-acetylglucosaminyl receptor sites on the cell surface will lead to an extremely strong interaction with the WGA immobilized on gel beads which will be difficult to break up by GlcNAc, whereas treatment of the cells with neuraminidase, thereby removing some of the binding sites or application of the cells in the presence of the inhibitor GlcNAc, improved the recovery of the cells from the columns considerably (Table V).

If the forces of interaction between glycoproteins and WGA are variable, then depending on the nature of the glycoprotein, it may be possible to fractionate mixtures of various glycoproteins on WGA column by elution with a gradient of the hapten sugar. We were able to obtain partial separation between fetuin or ovomucoid and glycophorin, since fetuin and ovomucoid apparently have weaker interactions with WGA in contrast to glycophorin as discussed above. This is another aspect which needs further investigation before developing it as a possible method for fractionation of cell membrane glycoproteins.

Greenaway and Levine (13) noted that approximately 9 mol of NeuNAc and 1 mol of GlcNAc were bound/mol of agglutinin when a 50-fold molar excess of the hapten over WGA was used. They concluded that, at this concentration, WGA had a higher affinity for binding NeuNAc than GlcNAc. Under optimum conditions, a maximum of 12 mol of NeuNAc would be expected to bind/mol of WGA, since it is proposed that WGA has four binding sites/mol, each of which has three subsites. Jordan *et al.* (18) investigated the binding of GlcNAc and NeuNAc to WGA by proton magnetic resonance and found that $K_{assoc}$ was 238 $M^{-1}$ for GlcNAc and 560 $M^{-1}$ for NeuNAc. Adair and Kornfeld (4) provided evidence indicating that sialic acid residues are a component of the WGA receptor on human erythrocytes. Several studies (14, 15, 59) have indicated that treatment of cells with neuraminidase decreased their binding affinity for WGA and also abolished WGA-induced agglutination. Not all cells are affected this way since neuraminidase digestion had no effect on WGA-induced agglutination of Novikoff tumor cells (60). WGA-resistant clones isolated from Chinese hamster ovary cells have shown defects in the sialylation of surface glycoproteins (5, 6). These and other reports (16, 17, 19) are consistent with our results.

In conclusion, the data provided in this paper clearly show that the *N*-acetylneuraminic acid residues of sialoglycopro-

teins are directly involved in specific interaction with WGA. Thus, it is likely that, in the interaction between the cell surface and WGA, the nonreducing terminal NeuNAc residues of glycoproteins and glycolipids play a more important role rather than the usually internal GlcNAc residues. Further, it is clear from our data that the topography of the NeuNAc and GlcNAc residues on the glycoconjugates and the cell surface is important for the formation of a stable association between the lectin and the glycoconjugates or the cell.

*Acknowledgments*—We wish to thank Mr. Jeffrey Kemper and Ms. Louise Morgan for technical assistance, Mrs. Joy Sizgorich for secretarial assistance, and all those listed under "Experimental Procedures" for their gifts of the various samples used in our studies. We also thank Dr. E. A. Davidson for his support and encouragement and his critical reading of the manuscript.

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STUDIES ON THE AGGLUTININ AND ON ENZYMIC PROTEINS ISOLATED FROM WHEAT GERM. V.P. Bhavanandan and Anne W. Katlic, Department of Biological Chemistry, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033.

We have previously reported the involvement of N-acetyl neuraminic acid in the interaction of wheat germ agglutinin (WGA) with sialoglycoproteins (1).

Multivalent nature of the interaction between sialoglycoconjugates and WGA:

In the present studies we purified WGA by affinity chromatography on a column of Fetuin glycopeptide Fraction C-Sepharose 4B. Fetuin glycopeptide Fraction C has the structure (NeuNAc→Gal→GalNAc-O-Ser/Thr peptide) (2) and does not inhibit hemagglutination by WGA and does not bind to WGA-Sepharose 4B columns (1). Thus the binding of WGA to this glycopeptide immobilized on Sepharose 4B gels is of interest and supports our postulate of the requirement of multivalent interaction between the ligands and WGA for the formation of stable complexes. To determine the minimum valency required for stable interaction between sialyl glycoconjugates and WGA, the binding of glycopeptides A and B isolated by CNBr treatment of glycophorin (3) was tested. The larger glycopeptide B containing the bulk of the sialic acid is completely bound, but only about 60% of the smaller glycopeptide A was bound; the balance is retarded (Figure). Of all the glycoconjugates so far tested the glycophorin CNBr glycopeptide A is the smallest molecule that bound to WGA affinity columns. Glycopeptide A is expected to contain 8 amino acids, 2 tetrasaccharides (NeuNAc→Gal→(NeuNAc)→GalNAc) and one trisaccharide (NeuNAc→Gal→Gal) (3). However, it is possible that the retarded portion of glycopeptide A has less sialic acid than the portion that bound to the column.

Role of internal GlcNAc residues in affinity binding to WGA-Sepharose 4B:

Affinity chromatography of [$^{14}$C]dimethyl $\alpha_1$-acid glycoprotein and [$^{14}$C]dimethyl asialoagalacto $\alpha_1$-acid glycoprotein on a WGA column resulted in the specific binding of 100% and 36% of the radioactivities, respectively. Previously, we have observed that whereas [$^{3}$H]acetyl- and [$^{14}$C]dimethyl fetuin were bound completely to WGA column, the corresponding asialo- and asialoagalacto fetuin preparations failed to bind to the same column. These results suggest that internal GlcNAc residues play only a minor role, if any, in contrast to terminal NeuNAc residues in the interaction of fetuin and $\alpha_1$-acid glycoprotein with WGA-Sepharose 4B.

Elution of glycoprotein bound to WGA-Sepharose 4B:

We demonstrated that glycoproteins bound to WGA columns could not be displaced by 1 M NaCl, 0.1 M NaOAc, pH 8.1 or 0.1 M acetic acid. In preliminary experiments, we observed that 10 mM NaOH (pH 11.7) was capable of displacing [NeuNAc-$^{3}$H]glycophorin from WGA columns. The significance of the elution by NaOH is not clear at the present.

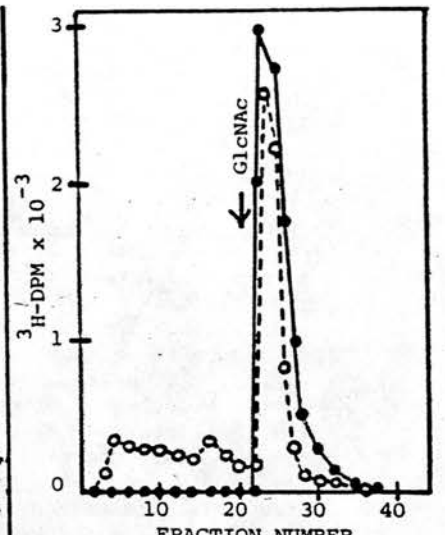
However, 0.1 M GlcNAc was the eluant routinely used to recover glycoproteins (even those lacking GlcNAc) from WGA columns. If sialic acid is one of the residues on glycoproteins for which WGA has affinity, then it should be possible to use this hapten inhibitor instead of GlcNAc to elute glycoproteins off a WGA column. Since column elution with sialic acid did not appear feasible due to economical reasons, we tested this possibility by batchwise elution in test tubes. The results showed that NeuNAc is capable of displacing glycoproteins from WGA-Sepharose gels even though batchwise elution by either NeuNAc or GlcNAc turned out to be very inefficient compared to the column elution technique.

Studies on Succinyl-Wheat germ agglutinin:

WGA is a basic protein, the isoelectric point of three of its isolectins being 8.7. We tested succinyl-WGA and found that it was incapable of agglutinating human and rabbit erythrocytes at concentrations

of 250 µg and 500 µg per ml respectively, whereas native WGA agglutinated both erythrocytes at 3 µg per ml. Removal of sialic acid and some of the surface negative charge by neuraminidase treatment rendered human erythrocytes agglutinable by succinyl-WGA at 62.5 µg per ml. In fact, treatment with trypsin which will remove sialoglycopeptides and peptides from the surface is even more effective in that trypsinized human and rabbit erythrocytes are agglutinated by succinyl-WGA at 1.0 µg and <2.0 µg per ml, respectively.

Succinyl-WGA, at 5 mg per ml, failed to produce a precipitin band with a large number of glycoconjugates in double diffusion experiments. Compounds tested included glycophorin and its glycopeptides, $\alpha_1$-acid glycoprotein, bovine and ovine submaxillary mucins, human lung mucin, fetuin all of which gave a precipitin line with native WGA at 2 mg per ml.



Affinity chromatography of glycophorin CNBr glycopeptides A (o---o) and B (●——●) on a WGA-Sepharose 4B column. Elution was with 50 mM Tris·HCl buffer, pH 8.0 followed by 0.1 M GlcNAc.

Succinyl-WGA-Sepharose 6MB was prepared by treatment of WGA-Sepharose 6MB with succinic anhydride. [NeuNAc-$^3$H]-glycophorin and [$^3$H]acetyl ovomucoid were not retained on the succinyl-WGA-Sepharose 6MB column. These results suggest that succinylation of the lectin, which converts it to an acidic protein, probably causes conformational changes, dissociation of the subunits etc., leading to the alteration of the carbohydrate binding capability of the lectin.

The isolation of an endochitinase from wheat germ extracts by affinity chromatography on chitin followed by gel filtration on Sephadex G-50 was reported by Molano et al. (4). Our attempts to separate chitinase from WGA on a Sephadex G-50 column were unsuccessful. However, we were able to separate an exo-β-hexosaminidase from endochitinase and WGA by the procedure described by Molano et al. (4). The endochitinase was isolated free of WGA by successive chromatography on columns of fetuin-Sepharose 4B, N-acetylated chitosan and finally Bio-Gel P30.

The relationship of the exo-β-hexosaminidase, the endochitinase and WGA are currently under investigation.

Acknowledgement: We wish to thank Drs. Michael Monsigny and Sally Bolmer for their kind gifts of succinyl-WGA and asialoagalacto $\alpha_1$-acid glycoprotein respectively. This work was supported in part by U.S.P.H.S. grant CA17686.

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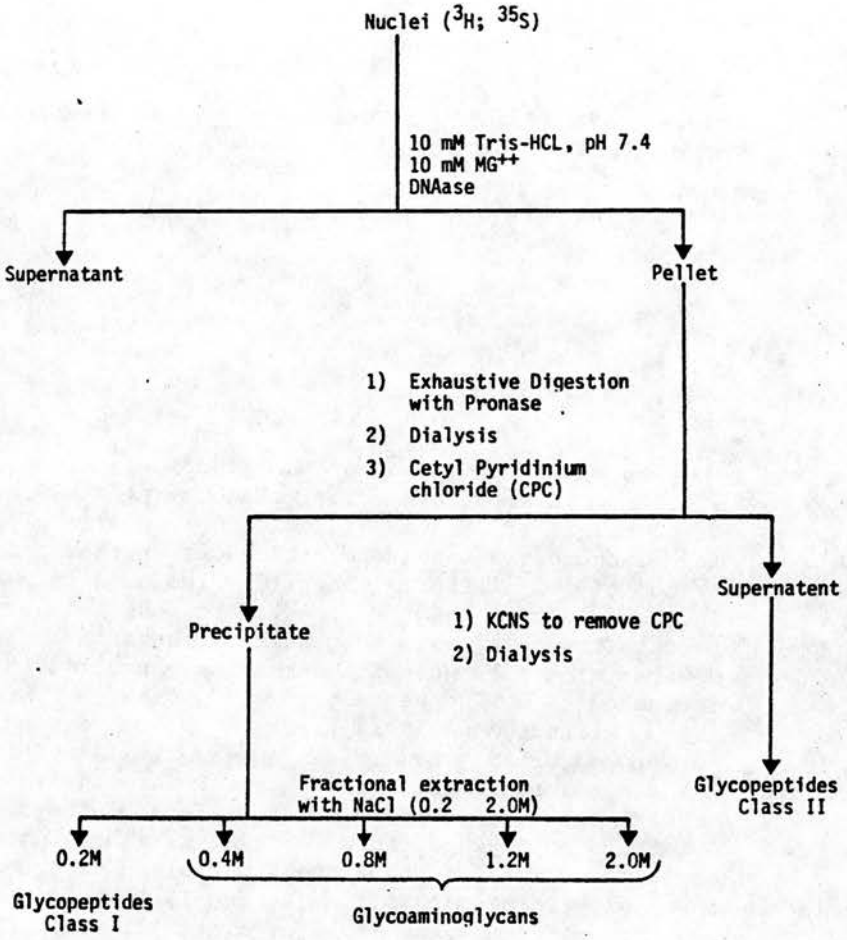
GLYCOCONJUGATE RESEARCH / Volume I
Proceedings of the Fourth International Symposium on Glycoconjugates

# Glycosaminoglycans and Sialoglycopeptides Associated with Mammalian Cell Nuclei

*Veerasingham P. Bhavanandan*

The characterization of the glycosaminoglycans associated with the nuclei of B16 mouse melanoma cells has been described previously (1). The major nuclear glycosaminoglycan component is a chondroitin 4-sulfate, which was further characterized by use of the milligram quantities isolated from spent media. The o.r.d. curve and i.r. spectrum of the melanoma chondroitin sulfate resemble those of cartilage chondroitin 4-sulfate. However, the mol. wt. of the melanoma chondroitin sulfate was estimated to be in the range of 90 000-120 000 by sedimentation equilibrium analysis, whereas the corresponding values for rib cartilage chondroitin sulfate is 12 000-15 000 (2). The minor nuclear glycosaminoglycans were characterized as heparan sulfate and chondroitin sulfate of lower mol. wt.

The sialoglycopeptides associated with the mouse melanoma nuclei were fractionated into two classes (I and II) by use of cetylpyridinium chloride (Scheme 1). The class I glycopeptides differed from those of class II, which are not precipitated with cetylpyridinium chloride, in greater molecular size and charge (sialic acid content) and affinity for wheat germ agglutinin. The distribution of the radioactivity in these glycopeptides was as follows: sialic acid (50%), galactosamine (43%), and glucosamine (7%) for class I; and sialic acid (36%), galactosamine (23%), and glucosamine


Nuclei ($^{3}H$; $^{35}S$)
10 mM Tris-HCL, pH 7.4
10 mM $MG^{++}$
DNAase
Supernatant
Pellet
1) Exhaustive Digestion with Pronase
2) Dialysis
3) Cetyl Pyridinium chloride (CPC)
Supernatent
Precipitate
1) KCNS to remove CPC
2) Dialysis
Glycopeptides Class II
Fractional extraction with NaCl (0.2 2.0M)
0.2M
0.4M
0.8M
1.2M
2.0M
Glycopeptides Class I
Glycoaminoglycans

*Scheme 1.*

(41%) for class II. About 85% of the radioactivity was in alkali-labile oligosaccharides in class I compared to 43% in class II. The sialoglycopeptides isolated from nuclei had a higher percentage of label in sialic acid than the sialoglycopeptides isolated from spent media. These preliminary data indicate that the nuclear sialoglycoproteins are richer in sialic acid and galactosamine as compared to the sialoglycoproteins present in the medium.

The nuclei isolated in our studies were free of obvious contamination by other organelles, as judged by phase-contrast and electron microscopy and by assays for marker enzymes (5'-nucleotidase, acid phosphatase, and succinic dehydrogenase). The nuclei, which were freed of the outer membrane, and therefore also of nonnuclear membrane contaminants, by treatment with 2.0% Triton X-100 (3), still retained between 70 to 80% of the initial radioactivity. Further, chromatin isolated from $^{3}H$- and $^{35}S$-labeled mouse melanoma nuclei yielded, on digestion with Pronase, the same complex saccharides as those obtained from the intact nuclei. These results indicate that glycosaminoglycans and sialoglycoproteins are integral components of the nuclei. In other control experiments, soluble glycoconjugates and membrane fractions prepared from cells labeled with [$^{3}H$]glucosamine and $Na_2{}^{35}SO_4$ were added to unlabeled cells prior to homogenization and isolation of nuclei. The results show that there was negligible adsorption of soluble glycoconjugates on the purified nuclei and that the contribution from nonnuclear membrane contaminants could not account for more than 10% of the radioactivity associated with the nuclei isolated by our procedures.

In preliminary studies, evidence for the presence of glycosaminoglycans associated with the nuclei of rat liver, Morris hepatoma 7777, human breast cells, and human breast cancer cells has been obtained. The presence of glycosaminoglycans associated with the nuclei of $HeLaS_3$ cells (4), rat brain (5), and human skin fibroblasts (6) has been reported. Although the significance of these findings remains to be determined, the present evidence strongly suggests that the association of glycosaminoglycans with the cell nuclei may be a general phenomenon.

ACKNOWLEDGMENTS

'I am grateful to Mr. Jeff Kemper for excellent technical assistance. This work is supported in part by USPHS Grant CA 17686.

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PURIFICATION AND PARTIAL CHARACTERIZATION OF HUMAN TRACHEOBRONCHIAL MUCIN GLYCOPROTEINS. V.P. Bhavanandan, B. Horsey and E.A. Davidson, Department of Biological Chemistry, The M.S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033.

The purification, characterization and partial structure of a high molecular weight glycoprotein from the bronchial secretions of an asthmatic patient has been described (1). In the present study we have examined a number of tracheal secretions from patients in pediatric and neonatal wards who have undergone surgery for non-pulmonary illness. The bronchial secretions were collected by sterile aspiration and the soluble components prepared as described previously (1). The high molecular weight glycoproteins, eluting at the void volume of Sepharose CL-4B columns, were isolated by gel filtration. The unfractionated samples and the high molecular weight fractions were analyzed for protein and carbohydrate contents. The analyses of a number of samples did not vary significantly. For detailed studies, the bronchial secretions of two patients (K.W. and C.L.), diagnosed as suffering from viral encephalitis (K.W.) and Guillain-Barré Syndrome (C.L.) were selected. The mucin glycoproteins from these secretions were isolated by gel filtration on Sepharose CL-4B (K.W.) or by precipitation with cetyl pyridinium chloride (C.L.). The carbohydrate and amino acid analyses of the isolated fractions are shown in the Table. The composition of K.W. peak I and C.L. 0.4 M NaCl fractions are typical of mucin glycoproteins. The fraction included in Sepharose 4B (K.W. peak II) and that remaining in the cetyl pyridinium chloride supernatent had analyses characteristic of serum glycoproteins. In double diffusion experiments, K.W. peak II gave precipitin lines with antisera against whole human serum, $\alpha_1$-acid glycoprotein and IgG.

The minimum molecular weight of K.W. peak I was estimated to be 1.4-3 x $10^6$ by equilibrium sedimentation ultracentrifugation. The results obtained indicate the presence of higher molecular weight species apparently resulting from aggregation of lower molecular weight components. The role of disulfide bonds as well as that of carbohydrate in the unusually high molecular size of the mucin glycoprotein was studied after labelling of the protein moiety by reductive methylation using $H^{14}CHO$ and $NaBH_3CN$ (2). The labeled glycoprotein was purified by rechromatography on Sepharose CL-4B. This removed low molecular weight contaminants, which were probably small amounts of proteins previously trapped in the aggregates of the mucin glycoprotein. The purified [$^{14}$C]dimethyl mucin glycoprotein was treated twice with dithiothreitol and iodoacetamide to reduce and block disulfide bridges. In a different approach, the labeled glycoprotein was treated with performic acid to oxidize the half-cystines to cysteic acid. The analysis of the performic acid treated sample is given in the Table. Chromatography of the reduced and carboxymethylated or the performic acid oxidized [$^{14}$C]dimethyl glycoprotein on Sepharose CL-4B columns showed that the molecular size was drastically reduced. In both cases the radioactive material was included in the column and major portions eluted in single peaks. Since the radioactivity in the treated samples was non-dialysable and eluted at the void volume of Sephadex G-50 columns, it was clear that label was still associated with macromolecules. These results indicate that disulfide bridges are one of the factors involved in the formation of the high molecular aggregate mucins.

The unlabeled as well as the [$^{14}$C]dimethyl labeled glycoproteins were treated with anhydrous hydrogen fluoride to cause deglycosylation without degradation of the peptide (3). The amino acid and hexosamine composition of the treated sample is shown in the Table. The amino acid composition was not significantly changed and >90% of the hexosamines were removed by the HF treatment. Gel filtration of the deglycosylated [$^{14}$C]dimethyl

glycoprotein on a Sepharose CL-4B column gave a well included single peak; on a Sephadex G-50 column the radioactive material eluted at the void volume. These results indicate that in addition to disulfide bridges, the carbohydrate residues of the mucin play an important role in the maintenance of the macromolecular structure and rheological properties of the mucin and hence of the bronchial secretion.

TABLE: Carbohydrate (% by dry weight) and amino acid (mole %) analysis of various fractions derived from bronchial secretions of two patients (K.W. and C.L.)

| | K.W. Sepharose 4B Peak I | | | C.L. Cetyl Pyridinium Chloride Fractions | |
|---|---|---|---|---|---|
| | Untreated | HF Treated | Performic Acid-Treated | 0.4 M NaCl | 2.0 M NaCl |
| $GlcNH_2$ | 10.0 | 0.6 | 7.2 | 16.0 | 12.0 |
| $GalNH_2$ | 4.0 | 0.0 | 4.0 | 8.0 | 5.0 |
| Neutral Sugars* | 34.0 | n.d. | n.d. | 39.0 | 42.0 |
| Sialic Acid+ | 4.0 | n.d. | n.d. | 2.0 | 3.0 |
| Cyst. Acid | 0 | 3.3 | 4.4 | 0.43 | 0.22 |
| Asp | 6.7 | 7.0 | 8.4 | 5.2 | 6.4 |
| Thr | 17.2 | 20.5 | 14.4 | 22.4 | 19.1 |
| Ser | 15.0 | 12.2 | 13.2 | 20.8 | 10.6 |
| Glu | 7.9 | 6.3 | 10.1 | 6.9 | 7.6 |
| Pro | 7.6 | 10.2 | 10.0 | 9.2 | 10.3 |
| Gly | 8.3 | 8.3 | 9.3 | 8.8 | 12.0 |
| Ala | 10.0 | 10.8 | 10.1 | 8.6 | 9.3 |
| Half-Cys | 5.4 | 0 | 0 | 2.6 | 5.5 |
| Val | 6.4 | 7.2 | 6.9 | 3.4 | 5.2 |
| Met | 1.9 | 0 | 0 | 1.5 | 0.88 |
| Ile | 3.5 | 4.5 | 3.5 | 2.3 | 2.4 |
| Leu | 6.0 | 5.8 | 7.2 | 4.7 | 6.8 |
| Tyr | 2.2 | 1.6 | 0 | 1.4 | 1.7 |
| Phe | 2.2 | 2.2 | 2.5 | 1.6 | 1.9 |

* Determined by Phenol-Sulfuric acid Assay using galactose.
\+ Determined by Thiobarbituric Acid method.

It is very likely that in general the high carbohydrate content of mucins, usually of the order of 80% by weight, play a similar role in maintaining the characteristics of mucus from such varying sources as submaxillary glands, gastro-intestinal, uro-genital and tracheo-bronchial tracts. The findings of Hill et al. (4) on the subunit structure of ovine submaxillary mucin are in support of this hypothesis.

Acknowledgement: This work was supported in part by U.S.P.H.S. Grant HL19190.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 11, Issue of June 10, pp. 5145–5153, 1980
*Printed in U.S.A.*

# Purification and Partial Characterization of a Murine Melanoma-associated Antigen*

(Received for publication, August 13, 1979, and in revised form, January 17, 1980)

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**A murine B16 melanoma-associated antigen was detected and partially purified from spent culture medium and intracellular material. The antigen was also purified and partially characterized from the detergent extract of cells cultured in the presence of [$^3$H]glucosamine alone or together with [$^{14}$C]leucine. During purification, the melanoma-associated antigen activity was monitored by a double antibody antigen binding assay (Bystryn, J.-C., Schenkein, I., Baur, S., and Uhr, J. W. (1974) *J. Nat. Cancer Inst.* 52, 1263). A radiochemically pure preparation of this glycoprotein antigen was obtained by gel filtration on Sepharose CL-4B and Sepharose CL-6B, ion exchange chromatography on DEAE-Sepharose, and chromatography on concanavalin A-Sepharose. Gel filtration on a calibrated Sepharose CL-6B column in the presence of detergent gave an apparent molecular weight of 375,000. Prolonged treatment with sodium dodecyl sulfate and 2-mercaptoethanol followed by gel filtration or acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated a subunit molecular weight in the range of 44,000 to 52,000. The tritium label in the antigen was distributed mainly between sialic acid and *N*-acetylglucosamine, with a small amount in *N*-acetylgalactosamine. Pronase digestion of antigen followed by fractionation on a Bio-Gel P-10 column yielded several glycopeptides. The results of the action of exo- and endoglycosidases on these glycopeptides and their behavior on lectin affinity columns suggested the presence of both complex (*N*-acetyllactosaminyl type) and simple (oligomannosyl type) oligosaccharides linked via *N*-acetylglucosamine to asparagine. The glycopeptide fractions isolated after proteolytic digestion were devoid of antigenic activity. Significant antigen activity was detected in the nuclei isolated from the labeled cells.**

It is widely believed that tumors possess unique tumor-associated antigens that are absent in normal adult tissues and which play an important but as yet not properly understood role in tumor growth. The isolation, purification, and characterization of these antigens is of paramount importance if their influence on tumor growth is to be elucidated.

The B16 murine melanoma has been extensively investigated (1–15), and it has been proposed as a model for human malignant melanoma (16, 17). Previous publications from our laboratories have reported on the nature of the glycosaminoglycans and glycopeptides isolated from B16 melanoma cells, culture media, and tumors (5–7, 18, 19). In addition, the presence of melanoma-associated antigens on the cells and their release into culture medium by viable cells was reported (8, 10). We have also shown that the partially purified antigens retained their biological activity since it was possible to immunize syngeneic mice with these antigens and inhibit the subsequent growth of otherwise lethal numbers of melanoma cells (11).

This study describes further purification and partial characterization of a radiochemically and immunologically pure melanoma-associated antigen.

## EXPERIMENTAL PROCEDURES

### *Materials*

The B16 mouse melanoma cell line described previously was used in these studies (7). Sepharose CL-4B and CL-6B and DEAE-Sepharose CL-6B were from Pharmacia. Bio-Gel P-2 and Bio-Gel P-10, both 200 to 400 mesh, and protein standards for gel electrophoresis were obtained from Bio-Rad. Pronase CB and *Vibrio cholerae* neuraminidase were from Calbiochem; leech hyaluronidase from Biotrics, Arlington, Mass.; endoglycosidase D and H (endo-$\beta$-*N*-acetylglucosaminidases), concanavalin A-agarose (glycosylex A), and protein standards for gel filtration were from Miles. Neuraminidase, endo-$\alpha$-*N*-acetylgalactosaminidase, $\beta$-hexosaminidase, and $\beta$-galactosidase from *Diplococcus pneumoniae* were isolated as described (20, 21). Concanavalin A, wheat germ agglutinin, *Ricinus communis* agglutinin 120 and 60 were isolated and conjugated to Sepharose 4B essentially as described (7). Concanavalin A-polyacrylamide was prepared as described by Lotan *et al.* (22).

Tritiated glucosamine (D-[6-$^3$H]glucosamine hydrochloride, 10 to 30 Ci/mmol) and $Na_2{}^{35}SO_4$ (carrier-free; 10 to 1000 mCi/mmol) were obtained from New England Nuclear. L-[U-$^{14}$C]Leucine (330 mCi/mmol) was from Amersham. [$^3$H]Acetylovalbumin glycopeptides were isolated as previously reported (21, 23).

Isotopically labeled and unlabeled glycophorin, $\alpha_1$-acid glycoprotein, fetuin, and ovomucoid were prepared by published procedures (24).

### *Methods*

*Cell Cultures*—The conditions of cell culture and of labeling of the complex saccharides produced by the cells have been described (7). For simultaneous labeling of saccharides and proteins, the cells were grown for 48 h in the presence of [$^3$H]glucosamine at 10 $\mu$Ci/ml and [$^{14}$C]leucine at 1.5 $\mu$Ci/ml of media.

The cultured cells were harvested at confluence by treatment with 0.02% EGTA[1] in NaCl/$P_i$. The cells were pelleted by centrifugation

* This investigation was supported by Research Grants CA 17686 and CA 13844 from the National Cancer Institute, National Institutes of Health, United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: EGTA, ethylene glycol bis($\beta$-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; MAA, melanoma-associated antigen; MAA-M, melanoma-associated antigen isolated from culture medium; MAA-C, melanoma-associated antigen isolated from cells; NP-40, Nonidet P-40 from Shell Chemical Corp., N. Y.; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin; Con A, concanavalin

(200 × *g*, 10 min) and washed thrice with serum-free media by resuspension and centrifugation.

*Antisera*—Rabbit antiserum to freshly excised murine B16 melanoma cells and rabbit IgG were prepared as described previously (8, 11). Prior to use, rabbit antimelanoma serum and normal rabbit serum were exhaustively absorbed with pooled normal syngeneic tissues (11).

The specificity of absorbed antimelanoma serum was examined by several methods (11, 25). The binding activity of the antiserum was reduced over 85% by quantitative absorption with increasing numbers of melanoma cells, whereas, it was reduced only 10% by equal numbers of syngeneic spleen cells under the same conditions. This finding indicates that the antiserum was not directed to H-2 or 1a antigens, which are strongly expressed on normal lymphoid cells. This was confirmed by the finding that binding activity was reduced by only 5% by absorption with a pool of normal allogeneic spleen cells prepared from different strains of mice which expressed most of the known murine transplantation antigens. B16 melanoma tissue prepared from surgically excised tumors absorbed binding activity as effectively as did cultured B16 melanoma cells, indicating that the antiserum was not directed to neoantigens, fetal calf serum, or other artifacts of cultured cells. By radioimmunoassay, the antiserum was shown not to react to the major structural protein.

*Assay for MAA*—MAA were identified and quantitated by a double antibody antigen binding assay. The assay is based on the coprecipitation by anti-Ig of immune complexes formed between excess antimelanoma antibodies and radiolabeled MAA (9). Briefly, triplicate 0.1-ml aliquots of material to be tested for MAA activity were diluted ¼ in NaCl/$P_i$ and incubated with 0.1 ml of antimelanoma or normal rabbit serum diluted 1/20 in NaCl/$P_i$. The assay was performed in the presence of 0.1 ml of fetal calf serum to reduce nonspecific binding of proteins. After 30 min of incubation at 37°C, antigen-antibody complexes were precipitated by the addition of goat anti-rabbit IgG. After an additional 30 min of incubation, 0.1 ml of normal rabbit serum diluted 1/10 in NaCl/$P_i$ was added to all tubes to provide carrier immunoglobulin, and the incubation continued for 30 min at 37°C and then for 18 h at 4°C. The precipitates were collected by centrifugation and washed three times with 3 ml of NaCl/$P_i$, dissolved in 0.5 ml of 0.25 N acetic acid, mixed with 10 ml of Beckman scintillation fluid and radioactivity was determined. Specific binding is defined as the radioactivity (counts/min) bound by antimelanoma serum less that bound by normal serum. The results are expressed as the percentage of added radioactivity bound specifically. In some cases the ratio of radioactivity bound by antimelanoma serum to that bound by normal serum, the binding index, was also calculated. Prior experiments have shown (9) that under these conditions there is a linear relationship between the amount of radioactivity bound specifically and the amount of labeled MAA present, indicating that the assay provides a quantitative measure of MAA concentration.

*Column Chromatography*—Sepharose CL-6B and CL-4B columns were equilibrated and eluted with Tris-HCl buffer, pH 8.0, with or without 0.1% Triton X-100. In preparative experiments 0.01% sodium azide and 0.1 mM of phenylmethane sulfonyl fluoride were included in the elution buffers. The elution profiles obtained in the presence of the protease and bacterial inhibitors were identical with those obtained in their absence. Bio-Gel P-2 and P-10 columns were equilibrated and eluted with 0.1 M pyridine acetate, pH 5.0. DEAE-Sepharose CL-6B was packed according to the manufacturer's instructions and regenerated to either the acetate or chloride form. After application of the sample, the column was eluted with a linear gradient of pyridine acetate, pH 5.0 (acetate form); NaCl or LiCl in Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 (chloride form). The recovery of radioactivity from the columns was between 80 and 100% unless otherwise specified.

*Lectin Affinity Columns*—The sample was applied and washed into the column which had previously been equilibrated with the elution buffer (50 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100). After being allowed to stand for about 15 min at room temperature, the column was eluted with the buffer followed by a solution of hapten sugar in the same buffer; the flow rate was controlled to about 10 to 15 ml/h. Fractions of 1 ml were collected and portions analyzed for radioactivity. The hapten sugar solution for the Con A column was methyl-α-mannoside (0.1 M unless otherwise indicated); for the WGA column, 0.1 M *N*-acetylglucosamine and for the RCA-120 and RCA-60 columns, 0.1 M lactose. The use of buffers containing $10^{-3}$ M $CaCl_2$, $MgCl_2$, and $MnCl_2$ (26) for the Con A column did not alter the results obtained.

*Determination of [$^3$H]Sialic Acid and [$^3$H]Hexosamines*—Total [$^3$H]sialic acid was determined either by acid hydrolysis (0.1 M $H_2SO_4$, 80°C, 1 h) or *V. cholerae* or *D. pneumoniae* neuraminidase treatment, followed by separation of sialic acid from asialoglycoprotein on a Bio-Gel P-2 column. In instances where the recovery of the asialoglycoprotein was poor on the Bio-Gel P-2 column, a Sepharose CL-4B column or a DEAE-Sepharose CL-6B column was used for the separation. An internal standard of unlabeled NeuAc or [$^{14}$C]NeuAc was included to establish the identity of product released by the acid or by the enzyme.

Hexosamine determinations on isotopically labeled compounds were carried out on acid hydrolysates (4 N HCl, 100°, 8 h) on an amino acid analyzer by a stream-splitting technique (5). Alternatively the hydrolysates were mixed with standard hexosamine and [$^{14}$C]-hexosamines and then chromatographed on a column of AG 50W-X8 according to Gardell (27); aliquots of the fractions were analyzed for hexosamine (28) and radioactivity.

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). The standard sugars were detected as described previously (7). Radioactivity on paper strips was estimated by extracting cut pieces with 1 ml of water in counting vials followed by scintillation counting. Liquid scintillation counting was performed on an Intertechnique model SL-36 spectrometer equipped with a DPM calculating module (7).

*Digestion with Glycosidases*—Digestions with neuraminidase (*V. cholerae* or *D. pneumoniae*), *D. pneumoniae* β-galactosidase, β-hexosaminidase, and endo-α-*N*-acetylgalactosaminidase were all done as described (7, 21). Treatment with endoglycosidase D or H was done in 50 mM citrate/phosphate (D, pH 6.5; H, pH 5.0) buffer at 37°C for 20 h (23).

Pronase digestion was performed in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $Ca^{2+}$ at 37°C for 120 h, in the presence of toluene and chloroform. Enzyme dissolved in the buffer was added at 0, 24, 48, and 96 h.

*Gel Electrophoresis*—Polyacrylamide gel electrophoresis was performed on 6.5%, 5.0%, and 4.0% gels in the presence of 0.1% sodium dodecyl sulfate as described by Weber and Osborn (29). Gel electrophoresis under nondenaturing conditions was performed similarly except that sodium dodecyl sulfate was omitted from the gels and in the buffers.

## RESULTS

*Isolation of Melanoma-associated Antigen from Culture Medium*—The medium (200 ml) was dialyzed at 4°C against 0.2 M NaCl for 2 days, followed by distilled water for 5 days, and then lyophilized. The powder was dissolved in NaCl/$P_i$ (200 ml), an equal volume of saturated $(NH_4)_2SO_4$ added and the pH adjusted to 8 with 1 M Tris base. After allowing the suspension to stand for 4 h at 4°C, the precipitate was collected by centrifugation, dissolved in 20 ml of 50 mM Tris-HCl, pH 8.0, and the resulting solution made 52% saturated by addition of solid $(NH_4)_2SO_4$. After standing overnight at 4°C the precipitate was recovered by centrifugation, washed with 52% saturated $(NH_4)_2SO_4$ by resuspension and centrifugation, and dissolved in 10 ml of 50 mM Tris-HCl buffer, pH 8.0. The percentage of radioactivity in the $(NH_4)_2SO_4$ precipitate specifically bound by antimelanoma serum varied from 2.1 to 7.4% in different preparations; the binding index was between 1.5 and 3.0.

*Purification of MAA-M*—The fractionation of the $(NH_4)_2SO_4$ precipitate of the culture medium on columns of Sepharose CL-4B is illustrated in Fig. 1. The material from each peak was tested for MAA activity with highest activity present in the void peak (Peak I). When the fractionation was repeated using buffer containing 0.1% Triton X-100, only negligible radioactivity eluted in the position of peak I (void

A; RCA-120 or RCA-60, *Ricinus communis* agglutinin molecular weight 120,000 or 60,000; NaCl/$P_i$, phosphate-buffered saline (0.14 M $Na^+$, 0.1 M $K^+$, 0.006 M $PO_4^{-3}$, pH 7.2); NeuAc, *N*-acetylneuraminic acid; GlcNAc, *N*-acetylglucosamine.

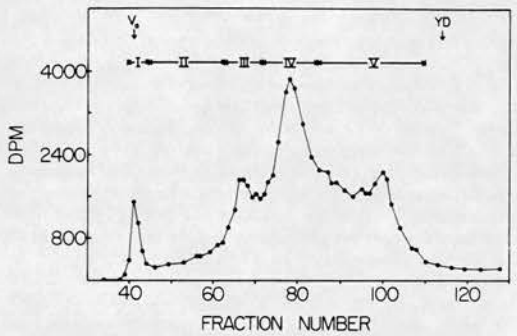


FIG. 1. **Fractionation of glycoproteins from media.** Fractionation of the glycoproteins isolated by ammonium sulfate precipitation from culture media on a column (2.7 × 110 cm) of Sepharose CL-4B using 50 mM Tris-HCl buffer, pH 8.0; 4.5-ml fractions were collected. Aliquots were analyzed for radioactivity. Fractions were combined as indicated to yield the numbered peaks. The combined fractions were concentrated to 2 to 10 ml in an Amicon concentration cell with a UM-10 membrane and dialyzed against 50 mM Tris-HCl buffer, pH 8.0, in the same cell. The peak elution positions of blue dextran ($V_0$) and yellow dextran (*YD*) are indicated by *arrows*.

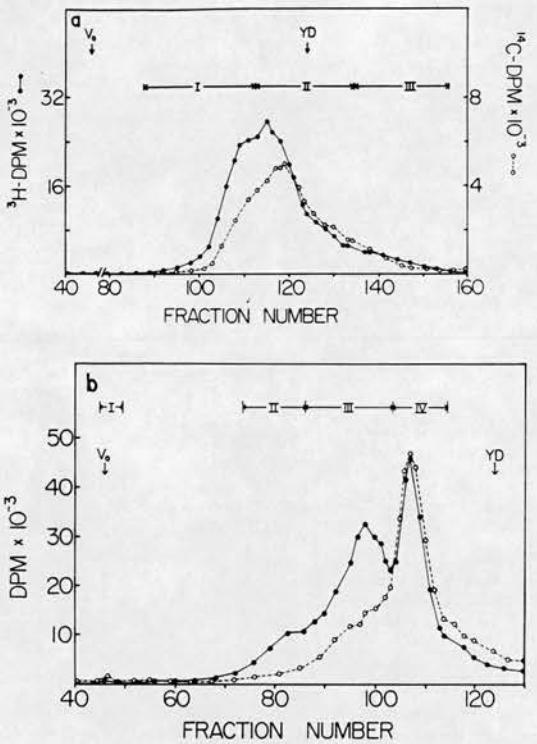


FIG. 2. **Fractionation of glycoproteins from cells.** Fractionation of the glycoproteins extracted by (*a*) 3 M KCl and (*b*) 0.5% NP-40 from B16 mouse melanoma cells metabolically labeled with [$^3$H]glucosamine and [$^{14}$C]leucine. The extracts were dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 and chromatographed in the same buffer on a column of Sepharose CL-4B (2.7 × 120 cm). Fractions of 4.2 ml were collected and aliquots analyzed for radioactivity. Fractions were combined as indicated to yield the numbered peaks, concentrated, and dialyzed against the elution buffer as described in Fig. 1. Peak elution positions of blue dextran ($V_0$) and yellow dextran (*YD*) are indicated by *arrows*.

volume). The elution profile was similar to that subsequently obtained with cell extracts (*cf.* Fig. 2*b*). Material with high MAA activity eluted in areas designated peaks II and III in Fig. 2*b*. Chromatography of peak II material on a Sepharose CL-6B column using Tris buffer containing 0.1% Triton X-100 resulted in further purification of MAA-M. Of the radioactivity in the main peak from the Sepharose CL-6B column 63% was specifically bound by antimelanoma serum and the binding index of this fraction was 38.2. The results of gel filtration in the presence of Triton X-100 suggest that the antigens found in peak I (Fig. 1) in the absence of detergent are aggregates. In agreement with this, when the glycoprotein from peak I (Fig. 1) was rechromatographed on Sepharose CL-4B using 50 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100, the radioactive antigenic material was essentially all included in the column, whereas if the rechromatography was carried out in buffer without Triton, then a portion of the material eluted at the void volume and the rest as a continuous trail (no sharp peaks).

*Detection of MAA in the Intracellular Material*—The EGTA supernatant obtained during harvesting of cultured labeled cells was exhaustively dialyzed at 4°C against 0.1 M NaCl for 2 days and against water for 5 days. The dialyzed solution was lyophilized, dissolved in Tris buffer, and tested for MAA activity. The percentage of radioactivity specifically bound by antimelanoma serum was 3.6%. Chromatography of the above material on a Sepharose CL-4B column gave an elution profile identical with that subsequently obtained with material in the NP-40 extract of cells (*cf.* Fig. 2*b*). The percentages of radioactivity specifically bound by antimelanoma serum were 9.0, 9.5, and 0.6% and the binding indices were 2.2., 2.3, and 1.3 for the material eluting in positions corresponding to peaks I, II, and III, respectively, in Fig. 2*b*. The melanoma antigens in the EGTA supernatant were not further investigated.

*Isolation of MAA from Cells*—The cell pellet was suspended in 3 M KCl (1.4 to 2.0 × $10^7$ cells/ml) and stirred at 4°C for 24 h. The resulting suspension was centrifuged at 64,000 × *g* for 60 min at 4°C, and the supernatant (KCl supernatant) saved. The pellet was then extracted with 0.5% Nonidet P-40 in 50 mM Tris-HCl, pH 8.0, at 4°C for 24 h and centrifuged as above. The KCl and NP-40 extracts were separately dialyzed against 6 liters of 50 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 at 4°C, the buffer was changed at 24 h and the dialysis stopped at 48 h. The dialyzed material was assayed for MAA activity. In a typical experiment, the percentage of radioactivity specifically bound by antimelanoma serum in the NP-40 extract was 2.8% compared to 0.7% for the KCl extract; the binding indices were 1.6 and 1.2, respectively.

*Purification of MAA-C*—Since Triton X-100 prevented the aggregation of MAA, all further chromatographic procedures were carried out using buffers containing 0.1% Triton X-100. The protease inhibitor, phenylmethane sulfonyl fluoride (0.1 mM), was also included in the buffer.

The KCl and NP-40 cell extracts were fractionated on Sepharose CL-4B columns, and individual fractions tested for MAA activity (Fig. 2 and Table I). Since the peak II material obtained from the NP-40 extract had the highest antigenic activity, it was further purified by sequential chromatography on columns of Sepharose CL-6B and DEAE-Sepharose CL-6B. The percentage of radioactivity in the purified material

TABLE I

*Properties of glycoproteins isolated from murine melanoma cells metabolically labeled with [$^3$H]glucosamine and [$^{14}$C]leucine*

| Sample | Radioactivity specifically bound by antimelanoma IgG | | Binding index | | [$^3$H]GlcNH$_2$/[$^3$H]GalNH$_2$ | Tritium activity specifically bound by WGA-Sepharose 4B |
|---|---|---|---|---|---|---|
| | $^3$H | $^{14}$C | $^3$H | $^{14}$C | | |
| | % | | | | | % |
| 0.5% NP-40 extract | 2.8 | 1.7 | 1.6 | 1.5 | ND[a] | 53 |
| After fractionation on Sepharose CL-4B | | | | | | |
| Peak I | 0.5 | 1.3 | 1.1 | 1.2 | ND | ND |
| Peak II | 27.4 | 16.2 | 19.4 | 17.7 | 4.6 | 90 |
| Peak III | 6.8 | 4.0 | 6.7 | 5.8 | 9.0 | 58 |
| Peak IV | 0.6 | 0.4 | 1.6 | 1.4 | 49.0 | 12 |
| 3 M KCl extract | 0.7 | 2.7 | 1.2 | 1.5 | | |
| After fractionation on Sepharose CL-4B | | | | | | |
| Peak I | 2.9 | 1.2 | 2.6 | 2.2 | ND | ND |
| Peak II | 2.1 | 0.6 | 2.0 | 1.5 | ND | ND |
| Peak III | 0.5 | 0.1 | 1.5 | 1.1 | ND | ND |
| NP-40 Sepharose CL-4B Peak II after purification on Sepharose CL-6B and DEAE-Sepharose | 76.8 | 68.7 | 70.7 | 29.6 | 14.0 | 92 |

[a] ND, not determined.

which was specifically bound by the antimelanoma serum, as well as the binding index, increased severalfold (Table I). This suggests that the combination of the chromatography on Sepharose 6B and DEAE-Sepharose may have removed some labeled nonantigenic contaminants and perhaps unlabeled contaminants capable of binding to the antibody. The material after gel filtration on Sepharose 6B but before chromatography on DEAE-Sepharose was not assayed for MAA activity. The percentage of label specifically bound by antimelanoma serum and the binding index of other purified preparations of MAA-C varied between 70 to 85 and 21 to 54, respectively.

*Nature of Labeled Contaminants in MAA and Attempts at Further Purification*—Chromatography of MAA-C on a calibrated column of DEAE-Sepharose CL-6B gave a single peak eluting at a concentration of 0.50 M LiCl. The peak elution positions of standards on this column were as follows: glycophorin, 0.505 M LiCl; hyaluronic acid, 0.55 M LiCl; heparan sulfate, 0.78 M LiCl; chondroitin sulfate, 1.05 M LiCl; and heparin, 1.25 M LiCl.

Since the antigen eluted close to the position of hyaluronic acid and had a high glucosaminé content, the preparation was examined for the presence of hyaluronic acid. Treatment of the antigen fraction with leech hyaluronidase (phosphate/citrate buffer, pH 5.6, 37°C, 24 h) followed by examination of the digest on controlled pore glass (6) and Sepharose CL-4B columns indicated that less than 5% of the radioactive material was degraded. The low molecular weight material produced eluted close to the position of glucuronic acid on the Sepharose 4B column and was not further characterized, but is presumably hyaluronic acid oligosaccharides. In an attempt to remove the glycosaminoglycans in the antigen preparations, MAA-C (Sepharose 4B Peak II in Fig. 2*b*) was treated with cetylpyridinium chloride in the presence of carrier anionic polysaccharides (6). Greater than 90% of the labeled glycoprotein material remained in the cetylpyridinium chloride supernatant but showed only 1.3% MAA activity, suggesting that the cetylpyridinium chloride treatment caused denaturation of the antigen.

On treatment of MAA-C with 8% trichloroacetic acid at 4°C, 82% of the radioactivity was precipitable. The precipitate showed only 6% MAA activity compared to about 60% in the starting material, suggesting inactivation of the antigen.

*Chromatography of MAA-C on Immobilized Lectins*—Chromatography on various concanavalin A-Sepharose or concanavalin A-acrylamide columns as described under "Experimental Procedures", results in recoveries varying from 22 to 32%. The reason for these poor recoveries is not clear. However, chromatography on a concanavalin A-acrylamide column at 37°C, instead of 25°C, and elution with a 5% solution of methyl-α-mannoside improved the recovery to about 80%. About 72% of the applied material passed through the column and was antigenically active.

Chromatography of MAA-C on WGA-Sepharose 4B and *Ricinus communis* agglutinin 120-Sepharose 4B columns resulted in 92 and 82%, respectively, of the radioactivity binding. However, the material eluted from these columns did not show any increase in MAA activity. Asialo-MAA, prepared by treatment of the purified material with neuraminidase, was poorly bound by the WGA-Sepharose (25%) but still interacted well with RCA-120 or RCA-60; 85% and 82% bound.

*Estimation of the Molecular Size of MAA-C*—Gel filtration of the purified MAA on a calibrated Sepharose CL-6B column using buffer with 0.1 or 1.0% Triton X-100 gave a single homogeneous peak (not illustrated). A semilogarithmic plot of molecular weights *versus* $K_{av}$ ($= (V_e - V_0)/(V_t - V_0)$) for glycoproteins with carbohydrate contents greater than 20% is linear (Fig. 3). Standards with lower carbohydrate contents (<10%) fall on a different straight line. Even though our results indicate that MAA-C is a sialoglycoprotein, we have no information on the percentage of carbohydrate in this molecule. If it is assumed that MAA-C has a carbohydrate content comparable to the glycoprotein standards falling on the linear plot, then MAA-C would have an apparent molecular weight of 375,000, whereas, if the carbohydrate content is low, a larger size is indicated.

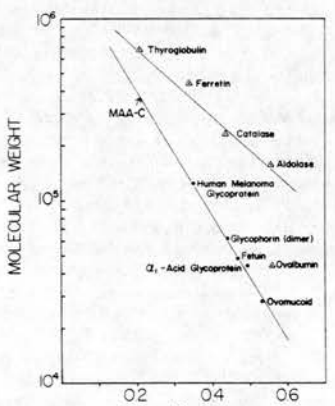


FIG. 3. **Molecular weight of MAA-C.** Estimation of the molecular weight of the purified MAA-C by gel filtration on a column (1.5 × 36 cm) of Sepharose CL-6B using 50 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100. The column was calibrated by chromatography of protein and glycoprotein standards together with [$^{14}$C]glucose as internal reference. Fractions were analyzed for protein (30) or radioactivity. Standards containing either no or low carbohydrate (△) behave differently from those containing high carbohydrate (●).

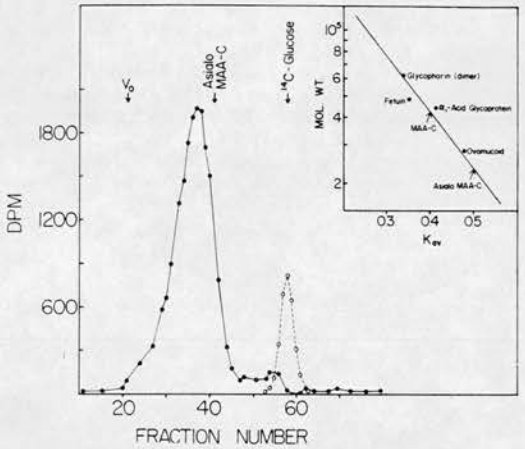


FIG. 4. **Gel filtration of MAA-C.** Gel filtration of MAA-C (●——●) after prolonged treatment with SDS and 2-mercaptoethanol (see text for conditions) and of [$^{14}$C]glucose (○- - -○) on a Sepharose CL-6B column (1.5 × 36 cm) using 50 mM Tris-HCl buffer, pH 8.0, containing 1% SDS and 10 mM 2-mercaptoethanol. The *inset* illustrates the calibration curve obtained with glycoprotein standards chromatographed under identical conditions. The peak elution position of asialo-MAA-C on the same column is also indicated.

Since gel filtration of MAA-M in aqueous buffer resulted in elution as an aggregate, we examined the possibility of further dissociating MAA-C. When the sample was treated with 1% sodium dodecyl sulfate and 10 mM 2-mercaptoethanol at 25°C for 30 min and rechromatographed on the Sepharose CL-6B column using buffer containing 1% SDS and 10 mM 2-mercaptoethanol, there was no significant alteration in its elution position or its $K_{av}$. However, prolonged incubation (64 h or more) with 1% SDS at 50°C followed by a 4-h incubation with 10% 2-mercaptoethanol as described by Kawasaki and Ashwell (31) resulted in dissociation of MAA into smaller subunits (Fig. 4). The apparent subunit molecular weight was 44,000 based on a calibration curve obtained by running the reference glycoproteins in buffer containing 1% SDS and 2-mercaptoethanol. It is known that glycoproteins behave anomalously in SDS systems (32) and this may be the reason for the four points of the standards not lying on a straight line (Fig. 4). In this system, glycophorin gave two peaks, a major one apparently corresponding to the dimer with $K_{av}$ 0.34, and a minor peak which is probably the monomer (33). A value of 23,500 was obtained for the apparent molecular weight of asialo-MAA, indicating that MAA has about 47% sialic acid. However, since it is known that sialoglycoproteins behave anomalously in the presence of SDS (32) and on gel filtration (34), other independent confirmations of the subunit molecular weight and sialic acid content are needed.

Electrophoresis of MAA-C using 6.5% or 5% acrylamide gels resulted in the sample failing to enter the gel; on 4% polyacrylamide gel the major portion of MAA-C entered the gel and had a mobility comparable to that of thyroglobulin. The samples, however, trailed instead of giving a sharp band.

Polyacrylamide gel electrophoresis of MAA in 5% gels in the presence of sodium dodecyl sulfate is illustrated in Fig. 5. A radioactive band was obtained corresponding to a molecular weight of 52,000 or 97,000 based on glycoprotein or pure protein standards, respectively, provided the sample was subjected to prolonged incubation with SDS and 2-mercaptoethanol. Shorter treatment with SDS (for example, heating for 10 min at 100°C) gave a band of radioactivity diffused over 6 cm of the gel (Fig. 5*a*).

*Sialic Acid and Hexosamine Analysis of MAA-C*—The proportion of sialic acid in the carbohydrate moiety of MAA-C was estimated by assessing the percentage of incorporated radioactivity released by mild acid hydrolysis (0.1 M $H_2SO_4$, 80°C, 1 h) or neuraminidase digestion followed by separation of the cleaved product by exclusion or ion exchange chromatography. The values ranged from 23 to 33%, probably reflecting some methodological variation. The identity of the monosaccharide released by neuraminidase treatment was confirmed as *N*-acetylneuraminic acid by chromatography on Dowex 1 (formate) followed by paper chromatography utilizing Solvent A.

The ratio of radioactivity in glucosamine to that in galactosamine was found to be 14.0, whereas NP-40-extracted Sepharose CL-4B peak II material had a ratio of 4.6 (Table I) suggesting the removal of galactosamine-rich contaminants.

*Isolation and Characterization of Glycopeptides from MAA-C*—The MAA-C was treated with pronase, the digest frozen and thawed three times, centrifuged at 1000 × *g* for 10 min and the glycopeptides in the supernatant fractionated by gel filtration on a Bio-Gel P-10 column (Fig. 6). The distribution of radioactivity in the major glycopeptide fractions is given in Table II.

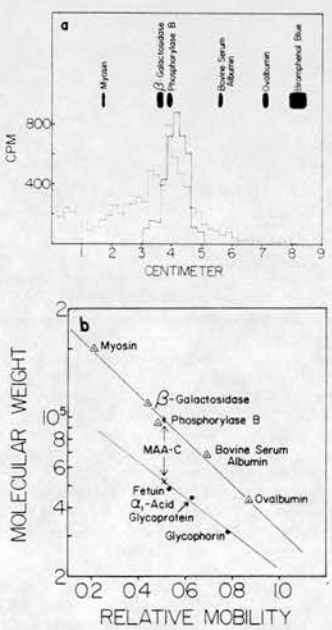


FIG. 5. **Gel electrophoresis of MAA-C in presence of SDS.** *a*, gel electrophoresis of MAA-C on a 5.0% polyacrylamide gel in the presence of SDS after treatment of the sample with 1% SDS at 100°C for 10 min (- - -) or at 50°C for 72 h (——). See text for further details. The gels were sliced into 2-mm segments, solubilized by incubation in glass vials with 0.2 ml of 60% perchloric acid and 0.4 ml of 30% hydrogen peroxide at 60°C for 4 h. Scintillation counting fluid was added to the solubilized gel and the radioactivity determined. The marker proteins were located by Coomassie blue G staining. *b*, calibration curves obtained with protein (△) and glycoprotein (●) standards on 5% polyacrylamide gels in the presence of SDS. The standards were treated with 1% SDS at 100°C for 10 min before electrophoresis.

These glycopeptides as well as glycopeptides produced by the treatment of antigen with trypsin did not have appreciable antigenic activity. In agreement with this, the Class I and II glycopeptides isolated from culture medium and cells (7) were also devoid of antigenic activity.

*Sialic Acid and Hexosamine Analysis of the Glycopeptides*—This distribution of tritium activity in sialic acid and hexosamines in the various glycopeptides is shown in Table II.

The chromatographic elution profiles of the asialoglycopeptides II and III on the Bio-Gel P-10 column are illustrated in Fig. 6.

*Treatment of Glycopeptides with Glycosidases*—The asialoglycopeptide III on treatment with *D. pneumoniae* β-galactosidase and β-*N*-acetylhexosaminidase released a labeled compound which was isolated by chromatography on a Bio-Gel P-2 column and desalted by passage through Dowex 50 ($H^+$) and Dowex AG-1 (formate) columns. On a paper chromatogram the major product had a mobility identical with that of GlcNAc. A minor product had a mobility similar to that of lactose and is probably the product of the endoglycosidase D activity present in trace amounts in the β-galactosidase used. The major product was further characterized as [$^3$H]GlcNAc by acid hydrolysis (3 N HCl at 100°C for 8 h) followed by detection of [$^3$H]$GlcNH_2$ on a paper chromatogram developed in Solvent System B. In control experiments treatment of asialoglycopeptide III with the hexosaminidase failed to release any labeled products.

The treatment of glycopeptide VI and standard [$^3$H]acetylovalbumin glycopeptides with endoglycosidase H followed

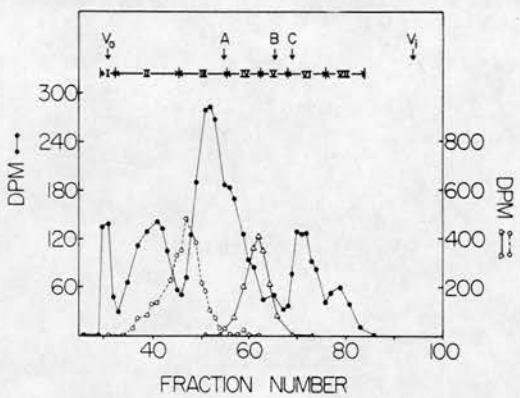


FIG. 6. **Fractionation of MAA-C glycopeptides on Bio-Gel P-10.** Fractionation of Pronase digest of MAA-C (●——●) on Bio-Gel P-10. The column (0.9 × 134 cm) was equilibrated and eluted with 0.1 M pyridine acetate, pH 5.0. Fractions of 1.0 ml were collected and aliquots analyzed for radioactivity. *Numbered areas* designate fractions which were combined and lyophilized to yield glycopeptides I to VII. Elution profiles of asialoglycopeptide II (○- - -○) and III (△——△) prepared by treatment with neuraminidase are superimposed. The peak elution positions of blue dextran ($V_0$), glucose ($V_t$), and fetuin glycopeptides A, B, and C (*A*, *B*, and *C*) are indicated by *arrows*.

TABLE II

*Properties of glycopeptides isolated from purified [$^3$H]MAA from cells by treatment with Pronase*

| Glycopeptide fraction | Distribution of radioactivity | Tritium activity in | | | Radioactivity specifically bound to | |
|---|---|---|---|---|---|---|
| | | Sialic acid | $GlcNH_2$ | $GalNH_2$ | WGA | Con A |
| | % | | % | | | |
| I | 4.0 | 45 | 41 | 14 | | |
| II | 19.0 | 50 | 14 | 36 | 0 | ND[a] |
| III | 36.0 | 28 | 66 | 6 | 0 | 0 |
| IV | 16.0 | ND | 98 | 2 | ND | 0 |
| V | 5.0 | | ND | | | |
| VI | 14.0 | 0 | 94 | 6 | 0 | 82 |
| VII | 6.0 | | ND | | | 85 |

[a] ND, not determined.

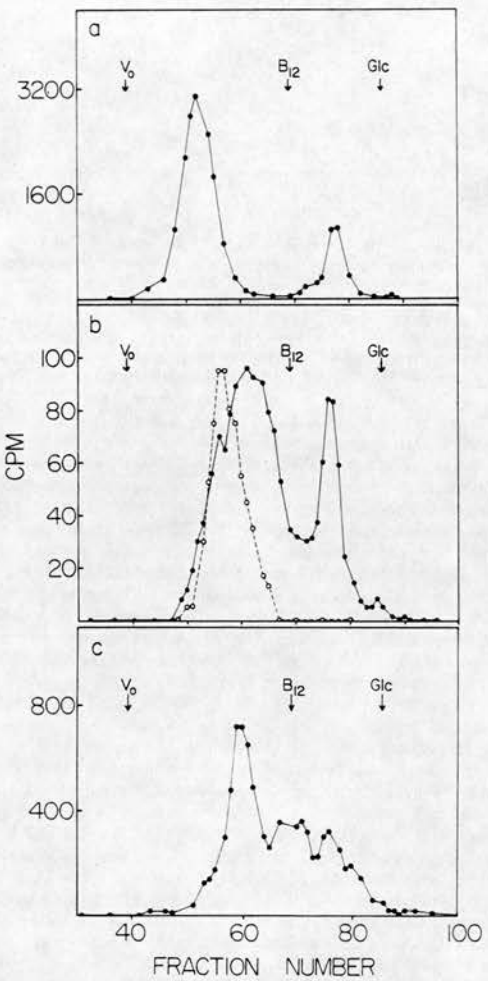


FIG. 7. **Gel filtration of glycopeptides treated with endoglycosidase H.** *a*, gel filtration of [$^3$H]ovalbumin glycopeptides after treatment with endoglycosidase H on a column of Bio-Gel P-4 (0.9 × 108 cm) using 0.1 M pyridine acetate. Untreated ovalbumin glycopeptides gave only the first peak. The elution position of the second peak was coincident with that of reference GlcNAc → Asn. *b*, gel filtration of MAA-C glycopeptide VI before (○- - -○) and after (●——●) treatment with endoglycosidase H under conditions identical with *a*. *c*, gel filtration of glycopeptide III which was first treated with neuraminidase, β-galactosidase, and β-hexasaminidase; reisolated and then treated with endoglycosidase H, on the same column as in *a*. Fractions of 1 ml were collected and analyzed for radioactivity in all cases. Peak elution positions of markers blue dextran ($V_0$), vitamin $B_{12}$ ($B_{12}$), and glucose (*Glc*) are indicated by *arrows*.

by gel filtration on a Bio-Gel P-4 column gave the results shown in Fig. 7. The treatment of asialoagalactoaglucosaminoglycopeptide III (see above) with endoglycosidase H gave several peaks, but only a minor peak eluted in the position of GlcNAc → Asn (Fig. 7*c*). Treatment of glycopeptide VI with endoglycosidase D did not result in the release of significant label.

*Affinity Chromatography of the Glycopeptides on Lectin-Sepharose Columns*—Glycopeptides VI and VII bound to a Con A-Sepharose 4B column and were specifically eluted with a 2% solution of methyl-α-mannoside. None of the glycopeptides showed affinity to a WGA-Sepharose 4B column, in contrast to the parent glycoprotein which was bound by this column (Tables I and II).

Asialoglycopeptide III bound to both *Ricinus communis* agglutinin 60- and 120-Sepharose 4B columns and was specifically eluted with 0.1 M lactose. The asialoglycopeptide III was subjected to hydrazinolysis (35) followed by chromatography on a Bio-Gel P-6 column; results are shown in Fig. 8.

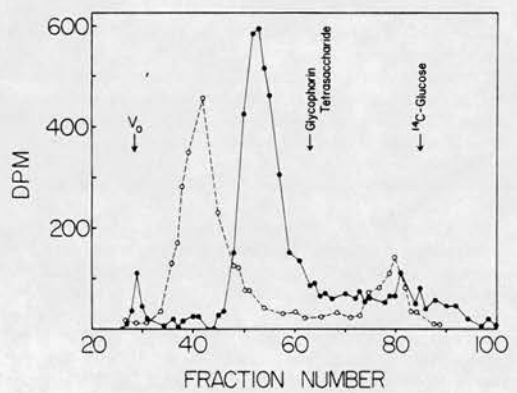


FIG. 8. **Chromatography of glycopeptides after hydrazinolysis.** Chromatography of asialoglycopeptide III (●——●) and [$^3$H]NeuAc $\alpha_1$-acid glycoprotein (○- - -○) on a Bio-Gel P6 column (0.9 × 105 cm) after hydrazinolysis. Fractions of 1.0 ml were collected and analyzed for radioactivity. Peak elution positions of references are indicated. Prior to treatment with hydrazine asialoglycopeptide III and $\alpha_1$-acid glycoprotein gave single peaks with peak positions at Fractions 45 and 29 (void), respectively.

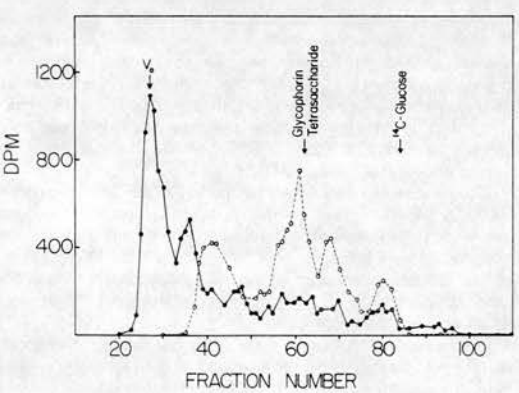


FIG. 9. **Gel filtration of MAA-C and glycopeptide II after borohydride treatment.** Gel filtration of MAA-C (●——●) and glycopeptide II (○- - -○) after treatment with alkaline borohydride on a Bio-Gel P6 column (0.9 × 105 cm). Fractions of 1.0 ml were collected and analyzed for radioactivity.

Treatment of glycopeptide II with alkaline borohydride (1 M $NaBH_4$-0.1 N NaOH, 37°C for 72 h) followed by neutralization and gel filtration on a Bio-Gel P-6 column indicated the presence of some oligosaccharides linked *O*-glycosidically to the peptide (Fig. 9). This is in agreement with the higher proportion of galactosamine in this glycopeptide fraction. Similar treatment of MAA-C resulted in the release of insignificant quantities of oligosaccharides (Fig. 9).

*Subcellular Location of MAA*—To obtain information on the localization of MAA in the cells, plasma membranes and nuclei were isolated from [$^3$H]glucosamine-labeled cells. The isolation procedure involved differential and sucrose gradient centrifugation which has been shown to yield plasma membranes and nuclei free of significant contamination by other organelles.[2] The membrane and nuclear pellets were then sequentially extracted with 3 M KCl and NP-40 detergent and dialyzed as described under "Isolation of MAA from Cells." The extracts were tested for MAA activity and the percentage of radioactivity specifically bound by the melanoma antisera were as follows: plasma membrane KCl extract, 3.9; plasma membrane NP-40 extract, 0.0; nuclei KCl extract, 5.8; and nuclei NP-40 extract, 6.3.

## DISCUSSION

Earlier studies indicated that some mouse melanoma-associated antigens are glycoproteins which were detectable both on the cells and in the culture medium (8, 10). We have now partially purified one of these antigen(s) and studied its biochemical properties.

Initial studies were conducted on the [$^3$H]glucosamine- and/or [$^{14}$C]leucine-labeled macromolecules released into the medium. A partially purified MAA was isolated by ammonium sulfate precipitation followed by gel filtration on Sepharose CL-4B and CL-6B. Column chromatography in the presence of detergents resulted in a marked shift of the antigenic material to lower molecular weight regions, suggesting that the MAA aggregated in the absence of detergents.

Examination of the material isolated from cells showed that the KCl-solubilized components were of lower molecular size and also had lower antigenic activity than the material subsequently extracted with NP-40 (Fig. 2 and Table I). It is possible that the material solubilized by KCl may have undergone partial proteolysis, since it has been demonstrated by Mann (36) that the release of histocompatibility antigens from lymphoid cells by 3 M KCl requires the presence of cell sap proteases. The 3 M KCl-extracted material was not further investigated. Subsequent treatment of the cell pellet after KCl extraction with 0.5% NP-40 solubilized over 90% of the labeled macromolecules in the pellet. The NP-40 extract was chosen for detailed studies of MAA because it had high quantities of the antigenic material and also was expected to have less fetal calf serum contaminants. Even though unlikely, we cannot completely exclude the possibility that the membrane-bound proteases in the cell pellet may have been active during the extraction with 0.5% NP-40 at 4°C and subsequent operations. Mann (36) found that only 3 to 6% of histocompatibility antigen was solubilized when the lymphoid cell membranes, free of cell sap, were extracted with 3 M KCl at 4°C for 24 h. Further, the treatment of MAA with Pronase or trypsin (see "Results") or with papain (8) completely abolishes its antigenic activity. Whereas MAA exhibited a macromolecular structure with a tendency to precipitate in the absence of detergents, the glycopeptides derived from MAA were easily

[2] J. Umemoto, J. G. Kemper, and V. P. Bhavanandan, unpublished results.

soluble in water and had molecular weights ranging from 4,000 to 12,000 (7). These data indicate that the MAA-C purified and characterized in this study has not been extensively degraded by proteolysis. The requirement of a detergent for solubilization from cells and aggregation behavior in the absence of detergents suggests that this macromolecule may be membrane-associated. However, MAA-C may not be the native form of the antigen present in the cell organelles (nuclei, membrane), and further studies are needed to resolve this problem.

Sequential chromatography of NP-40-extracted material having the highest MAA activity (Peak II, Fig. 2*b*) on Sepharose CL-6B, DEAE-Sepharose, and concanavalin A-Sepharose 4B columns gave apparently homogenous peaks of labeled material with increased specific antigenic properties.

Attempts to further purify the antigen were unsuccessful due to loss of antigenic activity. It was apparent from these results that both high pH (about 8.0) and detergent (0.1% Triton X-100) are essential to protect the antigenic properties of the isolated glycoprotein. Conditions of low pH, as encountered during chromatography using acetate buffer of pH 5.0, trichloroacetic acid precipitation, preparative isoelectric focusing, or desialylation with acid all resulted in the loss of antigenic activity.[3] Recognition by antibody was also significantly reduced in the absence of detergent probably due to precipitation of the antigen.

The MAA-C preparations appeared radiochemically homogenous when chromatographed on Sepharose CL-4B, Sepharose CL-6B, and DEAE-Sepharose (not illustrated). After prolonged treatment (72 h at 50°C) with SDS followed by gel electrophoresis in the presence of SDS a single but broad radioactive band was obtained. The band covers about 2 cm of the gel; however, 79 and 53% of the radioactivities fall within 1.0 and 0.6 cm of the gel, respectively. This diffuse band of 2 cm may be due to incomplete dissociation or partial reassociation of subunits. Electrophoresis of samples treated with SDS for shorter periods (10 min at 100°C (Fig. 5*a*) or 24 h at 50°C (not illustrated)) gave diffuse radioactive areas over 6 and 5 cm of the gel, respectively. An additional contribution to the gel behavior may be introduced by microheterogeneity in the carbohydrate moiety of the glycoprotein. The bulk (75 to 85%) of the labeled material in the purified MAA-C was specifically precipitated by an antiserum that reacted selectively with melanoma cells. These data suggest that our preparations consist of either a single glycoprotein exhibiting microheterogeneity or a group of antigenically related glycoproteins not separated by the above techniques. The presence of trace amounts of unlabeled material cannot be excluded even though other components were not detectable in column fractions (30) or gels (Coomassie blue staining). Since the characterization of MAA is based on the behavior of the labeled material, trace contaminants (if present) should not alter the major findings.

The antiserum used for monitoring the antigen had been raised to freshly excised B16 melanoma cells, a procedure which obviated the possibility of generating antibodies to neoantigens or other artifacts of cultured cells. On the basis of the specificity studies conducted to date with this antiserum (11, 25), the possibilities that the antigens it identifies are histocompatibility antigens, tissue-specific antigens of melanocytes, or one of the major type C viral proteins can be excluded. However, as in all studies of tumor antigens, the possibility that the purified antigen is simply one which is expressed to a greater degree on melanoma as opposed to unrelated cells cannot be completely discounted.

The molecular weight of MAA based on its mobility on a Sepharose CL-6B column in buffer containing 0.1 or 1.0% Triton X-100 was estimated to be about 375,000. Evidence for a strong association of monomer subunits even in the presence of 1.0% Triton X-100 was obtained when the sample was subjected to prolonged treatment with sodium dodecyl sulfate. Provided the MAA has a carbohydrate composition comparable to the glycoprotein standards used, values of 375,000 and 44,000 to 52,000 may be assumed for the native and subunit molecular weight, respectively. B16 mouse melanoma antigens having molecular weight in the range of 20,000 to 25,000 have been reported by Poskitt *et al.* (14). A more accurate determination of the molecular weight will be possible only after isolation of sufficient quantities of MAA for sedimentation equilibrium study and for quantitation of the carbohydrate content.

Some problems were experienced in determining the percentage of radioactivity present as sialic acid in MAA probably because of the insolubilization of the asialo-MAA in $H_2SO_4$ and/or the effect of the low pH (5.0) of the pyridine acetate buffer used to elute the column. Based on several experiments, we conclude that approximately 30% of the $^3H$ radioactivity in the MAA isolated from cells is in sialic acid and that virtually all of it is released as *N*-acetylneuraminic acid by *V. cholerae* neuraminidase. The balance of the radioactivity was distributed between glucosamine (64%) and galactosamine (6%).

The glycopeptides obtained by fractionation of a Pronase digest were investigated to obtain some information on the structure of the parent glycoprotein. Seven fractions were isolated, three of which contained significant levels of radioactivity in sialic acid; the $^3H$-label in other glycopeptides was mainly in $GlcNH_2$. Glycopeptides VI and VII interacted with concanavalin A-Sepharose and were cleaved by endoglycosidase H to release [$^3H$]GlcNAc → Asn (Fig. 7). This indicated the presence of an oligomannosyl core saccharide attached to the peptide backbone.

The results of the chromatography of the asialoglycopeptides II and III on a Bio-Gel P-10 column (Fig. 6) suggest that the differences between these two and the other glycopeptides were due to reasons other than different degrees of sialylation. This was further apparent in the high proportion of $GalNH_2$ ($GalNH_2/GlcNH_2 = 2.5$) in glycopeptide II compared to III. The release of oligosaccharides on treatment of glycopeptide II with alkaline borohydride was consistent with the high $GalNH_2$ content of this glycopeptide. However, the failure of the sialoglycopeptide II to interact with WGA-Sepharose 4B indicates the absence of clustered sialooligosaccharides (*O*-glycosidically linked to peptide via serine and threonine) such as those present in the Class I glycopeptides isolated from B16 mouse melanoma cells (7, 24).

The release of GlcNAc on treatment of asialoglycopeptide III with β-galactosidase and β-*N*-acetylhexosaminidase but not with β-*N*-acetylhexosaminidase alone indicates the sequence Gal → GlcNAc → in this glycopeptide. The presence of this sequence was also suggested by the interaction of the asialo-glycopeptide III and of the asialo-MAA itself with *Ricinus communis* agglutinin.

The release of oligosaccharide from the asialoglycopeptide III on hydrazinolysis and the susceptibility to endoglycosidase H of glycopeptide III which has been pretreated with neuraminidase, β-galactosidase, and β-*N*-acetylhexosaminidase indicated the presence of GlcNAc → Asn in this glycopeptide, as well.

In summary, we have succeeded in isolating an antigen associated with B16 mouse melanoma cells and purifying it to

[3] V. P. Bhavanandan, J. G. Kemper, and J.-C. Bystryn, unpublished results.

apparent homogeneity. The isolated antigen is a glycoprotein with a heterogenous population of oligosaccharides, which consist mainly of those terminating with the sequence NeuAc → Gal → GlcNAc→; some are terminated with mannosyl residues. These oligosaccharides, because of their susceptibility to endoglycosidase H and to hydrazinolysis, are presumably linked via *N,N'*-diacetylchitobiose to asparagine residues of the peptide chain. The presence of a few GalNAc-containing *O*-glycosidically linked oligosaccharides is indicated. The antigen was also partially purified from culture medium and some activity was associated with nuclei.

The information obtained in this study on the characteristics of MAA would enable us to isolate milligram quantities of this antigen from solid tumors (19) or from serum-free spent culture medium (18). Future studies will be directed toward the complete immunological and chemical characterization of this antigen.

*Acknowledgments*—We wish to thank Ms. Anne Katlic, Ms. Cory Ann Tedholm, and Mr. Michael Atchison for technical assistance; Mr. John Banks for help with cell culture; Mrs. Joy Sizgorich for typing the manuscript and Dr. E. A. Davidson for his interest in this work, support, and encouragement.

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# Glycosaminoglycans of Cultured Human Fetal Uveal Melanocytes and Comparison with Those Produced by Cultured Human Melanoma Cells†

V. P. Bhavanandan

ABSTRACT: The glycosaminoglycans produced by human fetal uveal melanocytes and by human melanoma cells were examined. The cells were grown in the presence of [$^3$H]glucosamine and [$^{35}$S]sulfate, and the labeled glycosaminoglycans were isolated from the cells, spent medium, and intracellular material. The distribution of the glycosaminoglycans was similar in both cells and spent media, which together accounted for 95% of the total. Of the total $^3$H-labeled glycosaminoglycans produced by the melanocyte culture, 42% was in chondroitin 4-sulfate, 25% in heparan sulfate, 16% in chondroitin 6-sulfate, and 17% in hyaluronic acid. In contrast, HM7 human melanoma cultures produced no chondroitin 6-sulfate, increased quantities of heparan sulfate, and less hyaluronic acid. A heparan sulfate fraction obtained from melanocytes required both heparitinase and heparinase for complete degradation, indicating the presence of heparin-like molecules in this fraction. The corresponding fraction from melanoma cells was totally degraded by heparitinase alone.

Several investigators have reported alterations in the glycosaminoglycans (Dietrich & Armelin, 1978; Dunham & Hynes, 1978; Hopwood & Dorfman, 1977; Glimelius et al., 1978; Keller et al., 1980; Klagsbrun, 1976; Muto et al., 1977; Vannucchi et al., 1978; Winterbourne & Mora, 1978) produced by malignant cells compared to normal cells. We have previously described the partial characterization of the glycosaminoglycans produced by cultured HM7[1] human melanoma cells and by a primary culture of human embryonic iris (Banks et al., 1976).

The subject of the present investigation is the study of the glycosaminoglycans produced by two established cell lines of human fetal melanocytes (Giovanella et al., 1976). In contrast to the primary explant of human embryonic iris, this system is a better control for the rapidly growing human melanoma cells. Even though the cells under comparison are of adult (melanoma) and fetal (normal melanocytes) origin, this is not undesirable since the detected differences would exclude the fetal antigens which are common to both tumor and fetal cells.

We describe below the characterization of the glycosaminoglycans produced by the cultured normal melanocytes. Additional characterization of the glycosaminoglycans isolated from human melanoma cells and culture media is also described. As in previous studies, the cell cultures were divided into three compartments: (i) the spent medium (medium), (ii) the intracellular material released by EGTA (EGTA supernatant), (iii) the material which remains associated with the viable cells (cell associated). The glycosaminoglycans of the normal fetal cells (iris and melanocytes) are compared to those produced by tumorigenic human melanoma cells. A preliminary report has been presented (Bhavanandan et al., 1979).

## Experimental Procedures

*Materials.* The human melanoma cell line, HM7, was isolated by Dr. John W. Kreider as described (Banks et al., 1976). The normal human uveal melanocyte cell lines FeMel 6 and FeMel 13 derived from embryonic tissues were kindly donated by Drs. McCormick and Giovanella, Cancer Research Laboratory, St. Joseph Hospital, Houston, TX (Giovanella et al., 1976). Components for culture media and fetal calf serum were obtained from Grand Island Biological Co. (Grand Island, NY) and Flow Laboratories (Rockville, NY), respectively. Pronase CB was from Calbiochem. Vitreous humor hyaluronic acid was obtained from Worthington (Freehold, NJ); chondroitin 4-sulfate was isolated from pig rib cartilage, and reference heparan sulfate was a gift from Dr. A. Linker (Salt Lake City, UT). Leech hyaluronidase (EC 3.2.1.36) was from Biotrics, Arlington, MA; bacterial chondroitinases ABC (EC 4.2.2.4), ACII (EC 4.2.2.5), and the unsaturated reference disaccharides ΔDi–4S, ΔDi–6S, and ΔDi–0S were from Miles Laboratories (Elkhart, IN). Heparitinase and heparinase were isolated from a *Flavobacterium heparinum* strain provided by Dr. A. Linker (Linker & Hovingh, 1972). All the above enzymes were free of contaminating mucopolysaccharidases when tested with isotopically labeled glycosaminoglycans as substrates. Glyceryl-controlled pore glass beads (glyceryl-CPG) were obtained from Electronucleonics (Fairfield, NJ). D-[6-$^3$H]Glucosamine hydrochloride (10–13 Ci/mmol) and $Na_2{}^{35}SO_4$ (carrier free; 80–800 mCi/mmol) were obtained from New England Nuclear (Boston, MA). Sources of other materials used in this study are given in the preceding paper in this issue (Bhavanandan et al., 1981).

*Methods. Column Chromatography.* Glyceryl CPG-240 beads of 80/120 mesh were packed with constant vibration and then equilibrated and eluted with 0.5 M KCl by using a pump to maintain a constant flow rate of 30 mL/h. For fractionation of glycosaminoglycans, DEAE-Sepharose CL-6B generated in the chloride form was used, and the column was eluted with a linear gradient of 0 → 1.5 M LiCl in 50 mM Tris-HCl buffer, pH 8.0.

*Enzyme Digestions.* Pronase digestion was performed in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $Ca^{2+}$ at 37 °C for 120 h, in the presence of toluene. Pronase CB dissolved in the buffer was added at 0, 24, 48, and 72 h. The

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[1] Abbreviations used: glyceryl-CPG, glyceryl controlled pore glass beads; CPC, cetylpyridinium chloride; AcNeu, *N*-acetylneuraminic acid; DEAE, diethylaminoethyl; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; FM6 and FM13, normal human uveal melanocyte cell lines FeMel 6 and FeMel 13; HM7, human melanoma cell line; Tris, tris(hydroxymethyl)aminomethane.

enzyme preparation used was free of contaminating exoglycosidases and endo-*N*-acetylhexosaminidase when assayed with *p*-nitrophenyl glycosides and [$^3$H]acetylovalbumin glycopeptides, respectively (Umemoto et al., 1977).

The labeled samples (10000–25000 dpm, $^3$H) were mixed with 500 μg of carrier glycosaminoglycans (hyaluronic acid, chondroitin sulfate, heparan sulfate, or heparin) and digested with the mucopolysaccharidases as described below. The carrier glycosaminoglycans were completely degraded under the conditions employed. Leech hyaluronidase digestion was carried out by dissolving the sample in 100 μL of McIlvaine's buffer (pH 5.6) and incubating at 37 °C for 6 h with addition of 125 μg of enzyme in 25 μL buffer at 0, 1, and 2 h. Chondroitinase digestions and paper chromatography of the products were performed according to the method of Saito et al. (1968). Heparitinase and heparinase digestions were done in 300 μL of 0.1 M sodium acetate buffer, pH 7.0, containing 1 mM calcium acetate for 24 h at 30 (heparinase) and 43 °C (heparitinase) (Linker & Hovingh, 1972). Blanks prepared as above but using heat-inactivated enzymes did not degrade the reference glycosaminoglycans.

Nitrous acid degradation was done by treating the sample in 100 μL of water with 20 μL of 3 M $NaNO_2$ and 20 μL of glacial acetic acid at room temperature (20 °C) for 80 min. Excess nitrite was destroyed by addition of 50 μL of 3 M glycine, and after 60 min at room temperature, the product was lyophilized.

The enzyme and nitrous acid degradation products were assessed by gel filtration on glyceryl-CPG-240 or Bio-Gel P-4 or P-6 columns.

Liquid scintillation counting was performed on an Intertechnique Model SL-36 or Model SL-4000 spectrometer equipped with disintegration per minute calculating modules. Usually 0.3–1.0-mL aqueous samples were mixed with 3–10 mL of counting liquid (ACS obtained from Amersham) in minivials or regular plastic counting vials, respectively. Efficiencies for $^3$H and $^{35}$S were about 25% and 65%, respectively, with a crossover of about 10% $^{35}$S into the $^3$H channel. When the external standard method was used, quenching was not detectable with any of the buffers.

Cellulose acetate electrophoresis was performed in a Beckman R-101 microzone electrophoresis cell by using 0.2 M calcium acetate (pH 7.0) at 5 mA for 3 h or pyridine/0.1 M formic acid bufer (pH 3.0) at 10 mA for 20 min. Reference glycosaminoglycans were detected by staining with Alcian blue (0.1% in 0.5% acetic acid). Radioactivity on cellulose acetate strips was estimated by dissolving cut pieces in 1 mL of glacial acetic acid in glass vials. After incubating at 37 °C for 3–6 h and cooling to room temperature, scintillation counting liquid was added, and the solution was mixed and counted. The recovery of the applied $^3$H and $^{35}$S radioactivities was between 80% and 90%.

Other methods used in this study are described in the preceding paper in this issue (Bhavanandan et al., 1981).

*Cell Culture.* The cells were propagated in 16-oz glass prescription bottles as described (Banks et al., 1976). When confluent, the cells were subcultured by using about $2 \times 10^6$ cells per bottle. The cultures were routinely tested for bacterial and yeast contamination as described previously (Banks et al., 1976). The cells were also tested for the presence of mycoplasma (Russell et al., 1975). No contamination was detected in the cells used in these studies.

Metabolic labeling of the glycoconjugates was accomplished by growing the cells in a medium containing isotopic precursors but no inorganic sulfate and one-third the usual glucose concentration. The FM6 and FM13 cells were labeled at the 17th and 20th passages, respectively. The isotopes used were [$^3$H]glucosamine and $^{35}SO_4^{2-}$ at 5 and 20 μCi/mL, respectively. The isotope-containing medium (25 mL) was added to logarithmically growing cells (4–8 bottles per experiment), and after 48 h, when confluency was reached, the cells were harvested. Exposure to labeled precursor for this time period is sufficient to provide an accurate estimate of mass ratios from incorporation levels. The medium was decanted, and the cell layer was rinsed 3 times with $NaCl/P_i$ buffer and then treated with 0.02% EGTA in $NaCl/P_i$ buffer, pH 7.2, at 37 °C for 10–20 min. The EGTA supernatant was removed after centrifugation, and the cell pellet was washed 3 times with the $NaCl/P_i$ buffer. The combined medium plus washings of the monolayers and the combined EGTA supernatant plus the washing of the cell pellet were again centrifuged to remove residual cells. The supernatants, referred to as spent medium and EGTA supernatant, respectively, and the cells were all separately examined for their labeled glycoconjugates.

The establishment and isotopic labeling of the human fetal iris explant were described previously (Banks et al., 1976). The labeled spent medium and the tissue (iris) were treated separately and analyzed for glycoconjugates. This system did not yield an EGTA supernatant fraction (extracellular material).

*Processing of the Cells, Spent Media, and the EGTA Supernatants.* The cells were extracted with chloroform/methanol (2:1 and then 1:2) as described by Hakomori & Murakami (1968). The pellet was treated with Pronase. The spent media and the EGTA supernatants were dialyzed at 4 °C against 0.15 M NaCl for 2 days, followed by distilled water for 4 days in the presence of toluene and chloroform. The nondialyzable materials were recovered by lyophilization and digested with Pronase.

*Separation of Glycopeptides and Glycosaminoglycans in the Pronase Digests by Treatment with Cetylpyridinium Chloride (Refer to Scheme I).* The Pronase digests were subjected to cetylpyridinium chloride precipitation as described (Bhavanandan et al., 1977). The glycosaminoglycans were recovered in the 0.2, 0.4, 0.8, 1.2, and 2.0 M NaCl extracts of cetylpyridinium chloride precipitate. The mixed glycopeptides present were isolated from the cetylpyridinium chloride supernatant by precipitation of the cetylpyridinium with KCNS followed by dialysis and lyophilization of the nondialyzable material. Table I presents the distribution of the radioactivity in the glycosaminoglycan and glycopeptide fractions.

*Fetal Calf Serum–[$^3$H]Glucosamine Control Experiment.* This experiment was designed to determine the contribution, if any, to the glycoconjugate pool of spent medium, of artifacts arising from the interaction of [$^3$H]glucosamine with fetal calf serum components. Fetal calf serum (100 mL) from the same batch used for metabolic labeling of cells was mixed with 400 μL of [$^3$H]glucosamine containing 400 μCi, and the mixture was incubated at 37 °C for 48 h. Triplicate aliquots were taken for determining radioactivity, and the balance was dialyzed at 4 °C against 0.15 M NaCl followed by water, two changes of 6 L each for a total of 8 days. When no further radioactivity was detectable in the dialysate, the dialysis was stopped. The nondialyzable material contained 0.76 μCi of radioactivity, which is 0.19% of the original. Two 2-mL aliquots were hydrolyzed (6 N HCl, 110 °C, 24 h), and the hydrolysate was purified on a column of Dowex 50 ($H^+$) (Boas, 1953) and analyzed on an amino acid analyzer by the stream-splitting technique. About 68% of the radioactivity coeluted with standard glucosamine. The balance of non-

Scheme I: Isolation of Glycosaminoglycans Produced by HM7 Human Melanoma Cells

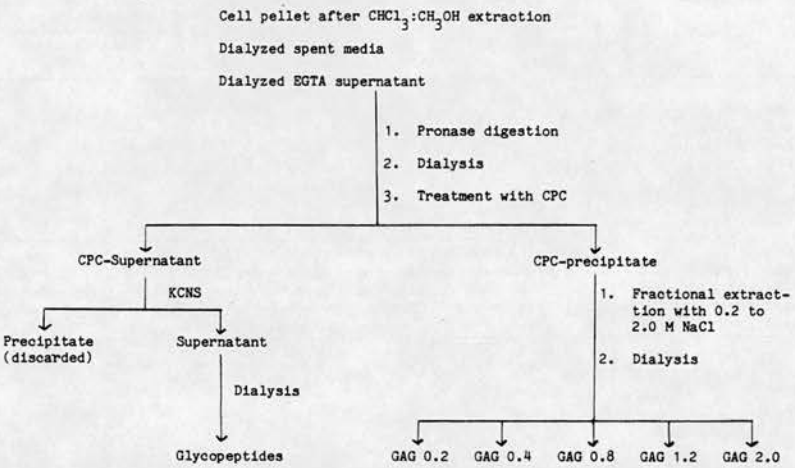


Table I: Distribution of $^3H$- and $^{35}S$-Labeled Glycoconjugates Produced by Human Fetal Uveal Melanocyte and Melanoma Cell Culture[a]

| | human fetal uveal melanocytes | | | HM7 human melanoma | | |
|---|---|---|---|---|---|---|
| | cell | spent medium | EGTA supernatant | cell | spent medium | EGTA supernatant |
| | | | glycopeptides | | | |
| $^3H$ | 26.52 (95.8) | 14.63 (56.2) | 16.61 (70.5) | 54.18 (94.9) | 34.78 (85.6) | 4.79 (92.2) |
| $^{35}S$ | 0.13 (8.0) | 0.84 (4.4) | 0.80 (8.7) | 0.13 (6.1) | 0.44 (5.7) | 0.03 (10.0) |
| | | | glycosaminoglycans | | | |
| $^3H$ | 1.17 (4.2) | 11.38 (43.7) | 6.95 (29.5) | 2.91 (5.1) | 5.85 (14.4) | 0.39 (7.8) |
| $^{35}S$ | 1.49 (92.0) | 18.32 (95.6) | 8.41 (91.3) | 2.04 (93.9) | 7.32 (94.3) | 0.26 (90.0) |

[a] The results are expressed as $^3H$ and $^{35}S$ radioactivity ($10^{-6}$ dpm$10^7$ cells) incorporated into the clycoconjugates. The numbers within parentheses are percentages relative to the total radioactivity which is the sum in the glycopeptides and the glycosaminoglycans in the respective culture compartments. The cells were grown in the presence of [$^3H$]glucosamine and $Na_2{}^{35}SO_4$ for 48 h during log phase of growth. The culture compartments (cell, medium, and EGTA supernatant) were digested with Pronase and dialyzed. The Pronase digests were treated with CPC to fractionate the glycopeptides and glycosaminoglycans. For details see Methods and Scheme I.

dialyzable material was digested with Pronase, and the digest was dialyzed exactly as for Pronase-digested spent medium. The nondialyzable material after Pronase digestion contained only 10.2% of the radioactivity present before the treatment. The nature of this radioactive material (108 000 dpm), 0.019% of the original, was not further investigated.

## Results

*Culture Characteristics of the Fetal Melanocytes.* The FM6 and FM13 cells grew as nonpiling monolayers of elongated cells compared to the multilayered foci growth of the HM7 human melanoma cells (Figure 1). A brown–black pigmentation was observed in the confluent monolayers of the melanocytes, which is indicative of the production of melanin by these cells. The doubling time of FM6 cells was determined to be 58 h whereas the HM7 cells had a doubling time of 40 h. The doubling times of three other human fetal uveal melanocytes are reported to be 62 (Mel 1B), 52 (Me 7–2A), and 108 (Mel 9) by Giovanella et al. (1976). In contrast to the HM7 cells which have been maintained in culture for over 4 years (>120 passages), both the normal melanocyte cell cultures, FM6 and FM13, failed to grow after about 30 passages.

*Tumorigenicity in Athymic (Nude) Mice.* Whereas HM7 cells produced large black tumor masses when $10^6$ cells were inoculated into nude mice, FM6 cells failed to produce tumors even when $10^7$ cells were inoculated. In an earlier work, HM7 cells produced only small nodules in nude mice; the reason for

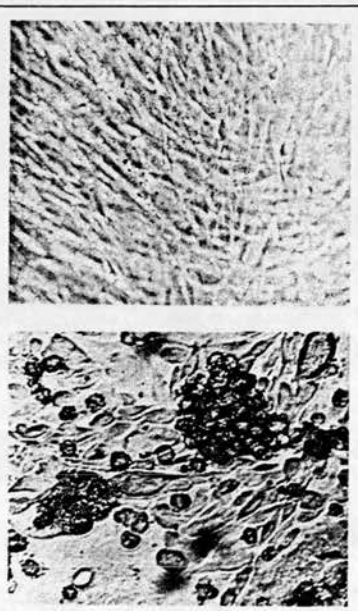

FIGURE 1: Photomicrograph of normal human uveal fetal melanocytes (top) and of HM7 human melanoma cells (bottom). Cultures grown to saturation. Phase contrast 100×.

Table II: Characteristics of GAG 0.8 Fractions[a]

| | [$^3$H]GlcNH$_2$ (%) | [$^3$H]GalNH$_2$ (%) | % low mol wt $^3$H after treatment with: leech hyaluronidase | heparitinase | chondroitinase AC | nitrous acid |
|---|---|---|---|---|---|---|
| cell | | | | | | |
| GAG 0.8 | 100 | 0 | 8 | 83 | NT | 96 |
| spent medium | | | | | | |
| GAG 0.8 | 88 | 12 | NT | NT | NT | NT |
| GAG 0.8a | 100 | 0 | 6 | 81 | 4 | 80 |
| GAG 0.8c | 91 | 9 | 0 | 88 | 10[b] | 89 |
| EGTA supernatant | | | | | | |
| GAG 0.8 | 97 | 3 | 0 | 100 | NT | 88 |

[a] Percent of radioactivity in the hexosamines was determined by hydrolysis (6 M HCl, 110 °C, 20 h) of the glycosaminoglycans followed by analysis by a stream-splitting technique on an amino acid analyzer as described previously (Bhavanandan & Davidson, 1976). The recovery of radioactivity was between 65 and 75%. The samples were treated as described under Methods with enzymes or nitrous acid, and the mixture was analyzed on Bio-Gel P-4 or P-6 columns. The percentage of radioactivity included in the column is given; the untreated samples were excluded from these columns in all cases. NT = not tested. [b] Paper chromatography showed both ΔDi-4S (65%) and ΔDi-6S (35%).

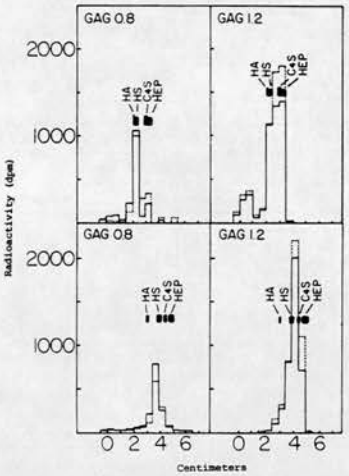


FIGURE 2: Cellulose acetate electrophoresis of human melanocyte glycosaminoglycan fractions GAG 0.8 and GAG 1.2. The human fetal melanocyte glycosaminoglycan fractions GAG 0.8 and 1.2 isolated from the spent medium of cultures labeled with [$^3$H]glucosamine and $Na_2{}^{35}SO_4$ were subjected to electrophoresis on cellulose acetate strips. Electrophoreses were carried out in 0.2 M calcium acetate, pH 7.0, at 5 mA for 3 h (top panels) and in pyridine/0.1 M formic acid buffer, pH 3.0, at 10 mA for 20 min (bottom panels). The radioactivity [$^3$H (—) and $^{35}$S (---)] and the reference glycosaminoglycans (HA, hyaluronic acid; HS, heparan sulfate; C4S, chondroitin-4-sulfate; and Hep, heparin) were detected as described under Methods.

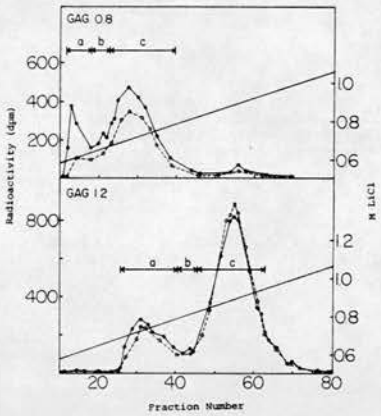


FIGURE 3: Fractionation of human melanocyte glycosaminoglycan fractions GAG 0.8 and GAG 1.2 on DEAE-Sepharose. The human fetal melanocyte glycosaminoglycan fractions GAG 0.8 and GAG 1.2 isolated from the spent medium of cultures labeled with [$^3$H]-glucosamine and $Na_2{}^{35}SO_4$ were chromatographed on a DEAE-Sepharose column (1 × 16 cm). The column was eluted with a linear gradient (total volume 100 mL) of 0.5–1.2 M LiCl. Fractions of 1 mL were collected and aliquots analyzed for radioactivity [$^3$H (●) and $^{35}$S (○)]. Material eluting in the area indicated by horizontal arrows marked a, b, and c were recovered by dialysis and lyophilization and designated GAG 0.8a, GAG 0.8b, etc. The GAG 1.2 fraction isolated from the EGTA supernatant was also similarly fractionated.

this is not clear (Banks et al., 1976). FM13 cells were not tested for tumorigenicity. Another fetal melanocyte cell line (Mel 7-2A) is also reported not to have yielded tumors when $10^7$ cells were injected subcutaneously and mice were observed throughout their life spans of 1–2 years (Giovanella et al., 1976).

*Characterization of the Glycosaminoglycans from Melanocytes*: *GAG 0.2 and GAG 0.4 Fractions*. These fractions from the three culture compartments all contained only [$^3$H]glucosamine and were totally excluded from the CPG column. Treatment with leech hyaluronidase followed by rechromatography on the CPG column indicated that the labeled material in these fractions was completely degraded. Ion–exchange chromatography on DEAE-Sepharose column, using a LiCl gradient for elution, of the GAG 0.2 (medium) and the GAG 0.4 (cell fractions) gave single peaks which coeluted with vitreous humor hyaluronic acid. Heparitinase had no effect on the fractions derived from the medium. Cellulose acetate electrophoresis of the GAG 0.2 (medium), GAG 0.4 (medium), and GAG 0.4 (EGTA) fractions gave single bands with mobilities identical with that of reference hyaluronic acid. Ion-exchange chromatography, heparitinase treatment, and cellulose acetate electrophoresis were not done on the other fractions due to insufficient material. However, on the basis of the results obtained, it was concluded that 0.2 and 0.4 M NaCl fractions from all three compartments contained essentially only hyaluronic acid.

*GAG 0.8 Fractions*. These fractions contained both $^3$H and $^{35}$S radioactivities. Cellulose acetate electrophoresis of the medium fraction in two buffer systems showed that the major component in this fraction had a mobility similar to that of heparan sulfate (Figure 2). The hexosamine composition (Table II), however, indicated the presence of both glucosamino- and galactosaminoglycans. The GAG 0.8 (medium) fraction was fractionated on a DEAE-Sepharose column (Figure 3), and three subfractions (a, b, and c) were obtained. The hexosamine composition of subfractions 0.8a and 0.8c is given in Table II; the minor intermediate fraction 0.8b was

Table III: Characteristics of GAG 1.2 Fractions[a]

| | [$^3$H]GlcNH$_2$ (%) | [$^3$H]GalNH$_2$ (%) | % low mol wt $^3$H after treatment with: heparitinase | heparitinase and heparinase | chondroitinase AC | nitrous acid |
|---|---|---|---|---|---|---|
| cell | | | | | | |
| GAG 1.2 | 38 | 62 | NT | 36 | 68 | NT |
| spent medium | | | | | | |
| GAG 1.2 | 24 | 76 | NT | NT | NT | NT |
| GAG 1.2a | 100 | 0 | 80 | 100 | 4 | 94 |
| GAG 1.2c | 0 | 100 | 0 | NT | 100[b,c] | 0 |
| EGTA supernatant | | | | | | |
| GAG 1.2 | 21 | 79 | NT | NT | NT | NT |
| GAG 1.2a | 100 | 0 | 63 | 98 | NT | 100 |
| GAG 1.2c | 0 | 100 | NT | 8 | 93[c] | NT |

[a] Details are as described in Table II. [b] Paper chromatography showed the presence of both ΔDi-4S (70%) and ΔDi-6S (30%). [c] Chondroitinase ABC digestion gave identical results.

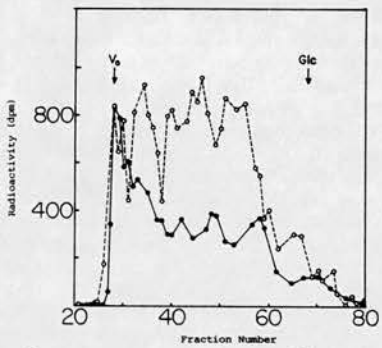


FIGURE 4: Chromatography on Bio-Gel P-6 of $^3$H-labeled glycosaminoglycan fractions from human fetal melanocytes after treatment with nitrous acid. The $^3$H-labeled glycosaminoglycan fractions GAG 0.8c (●) and GAG 1.2a (○) from spent medium of human fetal melanocyte cultures were treated with nitrous acid (see Methods for details) and chromatographed on a Bio-Gel P-6 column (0.9 × 105 cm). The column was eluted with 0.1 M pyridine/0.1 M acetic acid (pH 5.0) at 20 mL/h; 1-mL fractions were collected and analyzed for radioactivity. The void and included volumes of the column determined by the elution of blue dextran ($V_0$) and glucose (Glc) are indicated. The untreated glycosaminoglycans eluted at the void volume of the column. The results of the treatment of the various melanocyte glycosaminoglycan fractions with nitrous acid are summarized in Tables II and III.

not analyzed. The results of the treatment of GAG 0.8a and 0.8c (medium), GAG 0.8 (cell), and GAG 0.8 (EGTA) with leech hyaluronidase, heparitinase, chondroitinase, and nitrous acid are summarized in Table II. Typical results of the nitrous acid treatment are illustrated in Figure 4.

On the basis of the results, the major component of the GAG 0.8 fractions from all three compartments is characterized as heparan sulfate. The medium fraction, in addition, contained hyaluronic acid (2%) and chondroitin sulfate (12%), and the cell-associated GAG 0.8 contained about 8% hyaluronic acid. The galactosamine component (3%) of GAG 0.8 (EGTA) was not directly identified but is presumed to be chondroitin sulfate.

*GAG 1.2 Fractions.* These fractions had the largest percentage of $^{35}$S radioactivity, and the hexosamine analysis (Table III) suggested that they were mixtures of glucosamino- and galactosaminoglycans. Chromatography on a CPG column indicated components in the molecular weight range 15 000–40 000. Cellulose acetate electrophoresis of GAG 1.2 (cell) showed sulfated material moving as a broad band in the area of heparan sulfate and chondroitin sulfate (Figure 2). The medium and EGTA fractions were fractionated on DEAE-Sepharose to yield two major (a and c) and one minor (b) fraction each (Figure 3). The major fractions were found to have either glucosamine or galactosamine (Table III). The results of the action of nitrous acid, chondroitinase, and heparitinase (or heparitinase followed by heparinase) on these fractions are summarized in Table III. Typical results of chondroitinase treatment and the paper chromatographic pattern of the products are illustrated in Figure 5. Thus the components of the GAG 1.2 fractions from cell, medium, and EGTA supernatant are chrondroitin 4-sulfate (50%, 53%, and 59%), heparan sulfate (38%, 24%, and 21%) and chondroitin 6-sulfate (12%, 23%, 20%), respectively.

*GAG 2.0 Fractions.* The medium fraction on hydrolysis gave only galactosamine. It gave single peaks on both CPG and DEAE-Sepharose columns. On the basis of its mobility on the CPG column, the molecular weight of the component was estimated to be about 25 000. Chondroitinase AC and ABC completely digested the labeled material. The product was isolated by chromatography on a column of Bio-Gel P-4 and identified as ΔDi-4S by paper chromatography; no ΔDi-6S was detectable in contrast to the GAG 1.2 fraction (Figure 5, bottom). The GAG 2.0 (medium) fraction was not susceptible to either heparitinase or heparinase and was thus identified as chondroitin 4-sulfate. Insufficient material from the cell-associated and EGTA fractions was available for complete characterization. The 100% galactosamine composition of these two fractions, however, suggested that they consisted of chondroitin sulfate(s).

*Characterization of the Glycosaminoglycans from Melanoma.* Fractions GAG 0.4–2.0 which contain the glycosaminoglycans (hyaluronic acid, chondroitin sulfate, and heparan sulfate) have been partially characterized (Banks et al., 1976). Additional characterization of the melanoma glycosaminoglycans was carried out after further fractionation by chromatography on DEAE-Sepharose CL-6B columns and controlled pore glass as described above. Fraction GAG 0.2 from cell and medium was carefully examined since the corresponding fractions obtained from B16 mouse melanoma consisted mainly of a mucin-type sialoglycopeptide (Bhavanandan & Davidson, 1976; Bhavanandan et al., 1977). However, the human melanoma GAG 0.2 fractions on treatment with neuraminidase or mild acid released insignificant quantities of [$^3$H]sialic acid; further, the labeled material in these fractions did not interact with WGA–Sepharose 4B. Greater than 90% of the radioactivity in this fraction was identified as [$^3$H]glucosamine after acid hydrolysis; on treatment with leech hyalnuronidase, the labeled material was degraded to oligosaccharides. These results as

Table IV: Summary of the $^3H$-Labeled Glycosaminoglycans Produced by Human Fetal Uveal Melanocytes and Melanoma in Culture[a]

| glycan | human fetal uveal melanocytes | | | human melanoma | | |
|---|---|---|---|---|---|---|
| | cell | spent medium (48 h) | EGTA supernatant | cell | spent medium (48 h) | EGTA supernatant |
| hyaluronic acid | 2.78 (35.7) | 10.64 (13.8) | 8.41 (17.7) | 5.00 (15.8) | 8.24 (9.0) | 1.79 (28.0) |
| chondroitin 4-sulfate | 2.01 (25.8) | 35.15 (45.4) | 20.15 (42.5) | 13.76 (43.5) | 51.61 (56.4) | 2.53 (39.5) |
| chondroitin 6-sulfate | 0.41 (5.3) | 14.13 (18.3) | 6.38 (13.5) | 0 | 0 | 0 |
| heparan sulfate | 2.59 (33.2) | 17.43 (22.5) | 12.46 (26.3) | 12.85 (40.7) | 31.66 (34.6) | 2.08 (32.5) |

[a] The results are expressed as $^3H$ radioactivity ($10^{-5}$ dpm) incorporated into the various glycosaminoglycans obtained from the culture compartments cell, spent medium, and EGTA supernatant. The numbers within parentheses are the percentages relative to the total radioactivity present in glycosaminoglycans, in the separate culture compartments (vertical columns). Individual species of glycosaminoglycans were identified and quantitated as described under Methods and Results.

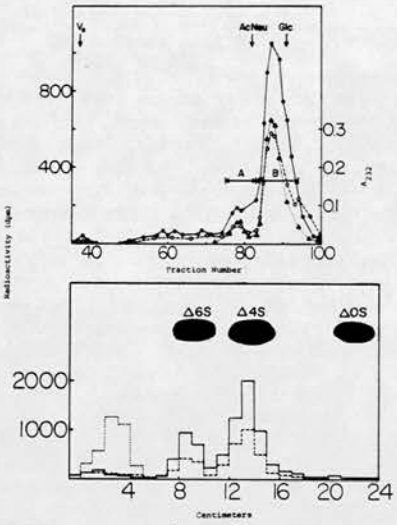


FIGURE 5: Chromatography of $^3H$- and $^{35}S$-labeled glycosaminoglycan fraction GAG 1.2c from human fetal melanocytes after digestion with chondroitinase AC on Bio-Gel P-4 (top) and paper (bottom). (Top panel) The $^3H$- and $^{35}S$-labeled glycosaminoglycan fraction GAG 1.2c isolated from human melanocytes was mixed with carrier chondroitin 4-sulfate treated with chondroitinase AC (see Methods for details) and chromatographed on a Bio-Gel P-4 column (0.9 × 110 cm). The column was eluted with 0.1 M pyridine/0.1 M acetic acid (pH 5.0) at 20 mL/h, and 1-mL fractions were collected. After the fractions were assayed for unsaturated disaccharides by measuring absorbance at 232 nm (▲), aliquots were analyzed for radioactivity [$^3H$, (●) and $^{35}S$, (○)]. Untreated GAG 1.2c and chondroitin 4-sulfate or controls prepared by treatment of the glycosaminoglycans with heat-inactivated enzyme eluted at the void volume ($V_0$). The peak elution positions of *N*-acetylneuraminic acid (AcNeu) and glucose (Glc) are indicated by arrows. The radioactive material eluting in the area marked by horizontal arrows A and B were recovered by lyophilization and examined by paper chromatography. (Bottom panel) The radioactive materials A [$^3H$, (···)] and B [$^3H$ (—) and $^{35}S$ (- - -)] were chromatographed on paper together with standard unsaturated disaccharides (ΔDi-4S, ΔDi-6S, and ΔDi-0S) by using the solvent system 1-butanol/acetic acid/1 M $NH_4OH$ (2:3:1 v/v). The migration positions of the standard unsaturated disaccharides were detected by examining the paper in UV light. Radioactivity on paper strips was estimated by extracting 1-cm cut pieces with 1 mL of water in counting vials followed by scintillation counting. The results of the treatment of the various melanocyte glycosaminoglycan fractions with chondroitinase AC followed by paper chromatography are summarized in Tables II and III.

well as those from cellulose acetate electrophoresis and DEAE-Sepharose chromatography confirmed that the human melanoma GAG 0.2 fractions from cells and spent medium were similar to the GAG 0.4 fractions which comprise hyaluronic acid.

The results of the characterization of the glycosaminoglycans produced by the human fetal uveal melanocyte and human melanoma cultures are summarized in Table IV.

## Discussion

The long-term cultures of the uveal melanocytes used in this study were derived from therapeutically aborted, 6–12-week human embryos by Giovanella et al. (1976). The cells were characterized as normal melanocytes on the basis of their ability to produce melanin and by their morphological characteristics (Giovanella et al., 1976). In our laboratory, we confirmed these observations on the FM6 and FM13 cultures. Further, in our studies, the FM6 cells failed to produce tumors in athymic mice under conditions which produced characteristic tumors from HM7 human melanoma cells. This together with the limited culture life (number of passage in culture) of the FM6 and FM13 cells compared to the virtual immortality of the HM7 human melanoma cells under identical culture conditions substantiates the nonmalignant nature of the fetal melanocytes.

[$^3H$]Glucosamine and $Na_2{}^{35}SO_4$ were employed as radioactive precursors to label the newly produced glycoconjugates. The possibility of labeled glucosamine interacting with fetal calf serum components of the culture medium leading to the formation of artifacts (Angello & Hauschka, 1974; Herrmann, 1974) was investigated in a control experiment. After [$^3H$]-glucosamine was mixed and incubated with fetal calf serum, exhaustive dialyses removed 99.81% of the radioactive material. The balance, identified as glucosamine, was apparently trapped or nonspecifically interacting with serum components since 90% of this dialyzed out following Pronase digestions. Thus the artifactual contribution to the pool of glycopeptides and glycosaminoglycans in the Pronase digest of the spent media in our experiments could be shown to be negligible (at the most, 0.019% of the added radioactivity). It should be noted that in the control experiment we used 10 times more glucosamine per milliliter of serum than that employed in the cell labeling experiments.

The total nondialyzable $^3H$ radioactivity in the Pronase digests of the melanocytes and melanoma cells did not differ significantly, but large differences were noted in the extracellular pools (the spent medium and EGTA supernatant). For example, the melanocytes incorporated 3.5 times more sulfate label into the extracellular (medium and EGTA supernatant) glycosaminoglycans than did the melanomas (Table I). A similar difference was noted in the synthesis of sulfated glycosaminoglycans by Balb/c 3T3 and SV40 Balb/c 3T3 cells (Klagsbrun, 1976). Further, the percent of CPC precipitable $^3H$ label (glycosaminoglycans) in the Pronase digests of the spent medium and EGTA supernatant was 14.4% and 7.8% for the melanoma culture and 43.8% and 29.5% in the case of the melanocyte culture (Table I). This indicates an increased secretion (or shedding) of glycosaminoglycans by the

melanocytes into the external environment, most of which was recovered in the GAG 1.2 fractions.

Whereas both melanocyte and melanoma cultures produced hyaluronic acid, chondroitin 4-sulfate, and heparan sulfates, only the melanocyte culture produced chondroitin 6-sulfate (Table IV). The cell, medium, and EGTA supernatant compartments of the melanocyte culture contained 5.3%, 18.3% and 13.5% of glycosaminoglycan-associated $^3H$ radioactivity in chondroitin 6-sulfate. Dermatan sulfate and keratan sulfate were not produced by either culture. There were quantitative differences in the production of hyaluronic acid, chondroitin 4-sulfate, and heparan sulfate by the two cell cultures. The distribution of $^3H$ in hyaluronic acid, heparan sulfate, and chondroitin 4-sulfate were 11.6%, 36.0%, and 52.4% for the melanoma culture and 16.5%, 24.5%, and 42.2% for the melanocyte culture (Table IV). An increased production of sulfated glycosaminoglycans, specifically heparan sulfate, with a concomitant decrease in hyaluronic acid production by malignant cells compared to normal counterparts has been previously observed (Goggins et al., 1972; Satoh et al., 1974; Chandrasekaran & Davidson, 1979). The glycosaminoglycans produced by the iris explant were markedly different from those produced by both of the continuous cell cultures (FM6 and HM7). In this case, about 79% of the $^3H$ radioactivity in the glycosaminoglycans was accounted for by hyaluronic acid, 14% by chondroitin 4-sulfate, 1% by heparan sulfate, and 5% by dermatan sulfate (Banks et al., 1976).

Several forms of heparan sulfate and chondroitin 4-sulfate appear to be present as indicated by the detection of these components in the 0.8, 1.2, and 2.0 M NaCl eluates of the CPC precipitate. Both heparinase and heparitinase were required to digest completely the heparan sulfate in the GAG 1.2 fractions (Table III) whereas heparitinase alone fully degraded the GAG 0.8 heparan sulfate (Table II). In comparison, the heparan sulfates produced by human melanoma culture were always completely degraded by heparitinase alone [Banks et al., (1976) and present results]. These data suggest that the heparan sulfate in the GAG 1.2 fractions of the melanocyte culture may have structural features resembling heparin, for example, a higher degree of N- or O-sulfation. The faster mobility on cellulose acetate electrophoresis of the glycosaminoglycans in GAG 1.2 compared to GAG 0.8 (Figure 2) and the nitrous acid degradation patterns of GAG 0.8c and GAG 1.2a (Figure 4) further supports this possibility. It has been shown that heparan sulfate isolated from normal Swiss 3T3 mouse cells contains 8% more *O*-sulfate than that isolated from SV40 transformed Swiss 3T3 cells (Keller et al., 1980).

Glycosaminoglycans which are major constituents of the cell surface and extracellular matrix are believed to be important in modifying the growth of cells both in vitro and in vivo. These molecules may be involved in the adhesion of cells to each other and to the substratum or membranes (Roblin et al., 1975; Culp et al., 1978; Turley & Roth, 1980).

A role of masking tumor antigens has also been postulated for the cancer cell surface glycosaminoglycans (Takeuchi et al., 1977). However, thus far the studies on the production of glycosaminoglycans by a wide variety of cultured cancer cells have given inconsistent results. For example, whereas increased sulfated glycosaminoglycan production by virus transformed fibroblasts has been reported by Goggins et al. (1972), Satoh et al. (1973), Makita & Shimojo (1973), and Hopwood & Dorfman (1977), others have reported a decrease in the production of these molecules by virus transformed cells (Saito & Uzman, 1971; Terry & Culp, 1974; Roblin et al., 1975). Similarly, both an increase (Temin, 1965; Satoh et al., 1973; Hopwood & Dorfman, 1977) and a decrease (Hamerman et al., 1965; Satoh et al., 1974) in the production of hyaluronic acid by malignant cells have also been recorded.

In the present studies, we have grown the cells in the same medium, labeled them at comparable cell densities and growth phase, and used the same concentration of isotopes in order to minimize the influence of external factors. Accordingly, the observed changes between the malignant and control (fetal) cultures should be considered characteristic of the cell type rather than due to artifactual reasons.

Acknowledgments

I am grateful to Drs. K. McCormick and B. C. Giovanella (St. Joseph Hospital, Houston, TX) for providing the human fetal melanocyte cultures which made this research possible. I also thank Dr. A. Linker for a gift of heparan sulfate, John Banks for invaluable assistance with tissue culture and tumorigenicity tests, and Anne Katlic and Jeff Kemper for expert technical assistance. The constant encouragement, support, and helpful criticism of the manuscript by Dr. E. A. Davidson is gratefully acknowledged.

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Reprinted from Biochemistry, 1981, *20*, 5586.


# Partial Characterization of Sialoglycopeptides Produced by Cultured Human Melanoma Cells and Melanocytes†

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ABSTRACT: The sialoglycopeptides produced by HM7 human melanoma and fetal uveal melanocyte cultures grown in the presence of [$^3$H]glucosamine and [$^{35}$S]sulfate were isolated from the Pronase digests of cells, spent media, and intracellular material. From the melanoma culture, six sialoglycopeptides, accounting for 43% of the total $^3$H radioactivity in the nondiffusible cell-associated glycopeptides, were purified. A major glycopeptide (GPIb) having an apparent molecular weight in the range 12000–15000 showed specific sialic acid dependent interaction with wheat germ agglutinin (WGA). It was found to contain mainly O-glycosidically linked oligosaccharides having the structure (AcNeu)$\rightarrow_{0\text{-}2}$[Gal$\rightarrow$GalNAc]; some N-glycosidically linked saccharides were also present. A second WGA-binding glycopeptide (GPIa) was smaller and less anionic and had a higher proportion of N-glycosidically linked saccharides than GPIb. The normal fetal cultures yielded either no (iris) or markedly reduced (melanocytes) quantities of the WGA-binding glycopeptides. The four WGA-nonbinding sialoglycopeptides purified from melanoma were shown to have complex (*N*-acetyllactosaminyl type) oligosaccharides linked via *N*-acetylglucosamine to asparagine with either no or insignificant amounts of O-glycosidically linked saccharides. The corresponding glycopeptides from melanocytes were of smaller molecular size and lower anionic charge, reflecting an overall lower degree of glycosylation.

Differences in the glycoproteins produced by malignant cells compared to normal cells have been reported (Bramwell & Harris, 1978; Buck et al., 1979; Ceccarini & Atkinson, 1977; Codington et al., 1979; Glick, 1979; Muramatsu et al., 1979; Takasaki et al., 1980; van Beek et al., 1977). A molecular description of these alterations is of importance because it will help to understand the biological properties of malignant cells.

We have investigated in some detail the glycoproteins and glycopeptides isolated from B16 mouse melanoma cells grown in culture and in vivo. The characterization of a mucin-type sialoglycopeptide (Bhavanandan & Davidson, 1976; Bhavanandan et al., 1977; Fareed et al., 1978) and a melanoma-associated antigen (Bhavanandan et al., 1980) have been reported. The characterization of a malignancy-related mucin-type sialoglycopeptide produced by human breast cancer cell lines has also been reported (Chandrasekaran & Davidson, 1979).

We report here the purification and partial structures of the major glycopeptides isolated from cultured human melanoma cells and from the culture media. These are compared to those produced by normal fetal melanocytes in culture. The glycopeptides obtained from the highly tumorigenic (in athymic mice) human melanoma cells are markedly different from those obtained from nontumorigenic human fetal uveal melanocytes. A preliminary report of these results has been presented (Bhavanandan et al., 1979).

## Experimental Procedures

*Materials.* The cells and culture conditions used are described in the following paper in this issue (Bhavanandan, 1981). Neuraminidase (*Vibrio cholerae*) was from Calbiochem. Bio-Gel P-2, P-4, P-6, and P-10 were obtained from Bio-Rad Laboratories (Richmond, CA). DEAE-Sephacel, DEAE-Sepharose CL-6B, Sephadex G-50, and Sephacryl S-200 were purchased from Pharmacia (Piscataway, NJ). β-Galactosidase, β-*N*-acetylhexosaminidase, and endo-α-*N*-acetylgalactosaminidase from *Diplococcus pneumoniae* culture filtrates were prepared in this laboratory. These enzymes were free of contaminating glycosidases as tested with *p*-nitrophenyl glycosides and isotopically labeled natural substrates (fetuin or pig submaxillary mucin glycopeptides) (Umemoto et al., 1977). Endo-β-*N*-acetylglucosaminidase D (Muramatsu et al., 1978) and endo-β-*N*-acetylglucosaminidase H (Tarentino & Maley, 1974) were purchased from Miles (Elkhart, IN). Highly purified exo-β-galactosidase from jack bean (Li et al., 1975) and endo-β-galactosidase from *Escherichia freundii*

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(Nakagawa et al., 1980) were kindly provided by Dr. Y.-T. Li of Tulane University (New Orleans, LA). Wheat germ agglutinin, concanavalin A, *Ricinus communis* agglutinins of molecular weights 120000 and 60000 ($RCA_{120}$ and $RCA_{60}$),[1] and soybean agglutinin were isolated and conjugated as described (Bhavanandan & Katlic, 1979a; Bhavanandan et al., 1977). [[$^3$H]AcNeu]glycophorin and its Pronase and tryptic glycopeptides were prepared as described (Bhavanandan & Katlic, 1979a). Fetuin glycopeptide fractions A, B, and C were isolated and labeled by reductive methylation with H$^{14}$CHO (Umemoto et al., 1977; Bhavanandan & Katlic, 1979a). Cyanogen bromide fragment A from [[$^3$H]AcNeu]glycophorin was prepared according to Blumenfeld & Puglia (1979).

*Methods. Column Chromatography.* Columns of Sephadex G-50 (fine), Bio-Gel P-2 (minus 400 mesh), P-4, P-6, and P-10 (200–400 mesh) were equilibrated and eluted with 0.1 M pyridine/0.1 M acetic acid, pH 5.0. DEAE-Sepharose CL-6B and DEAE-Sephacel columns were generated in the acetate form, packed in columns, and extensively washed with 0.1 M pyridine/0.1 M acetic acid, pH 5.0. The columns were finally equilibrated in 0.01 M pyridine/0.01 M acetic acid, pH 5.0, prior to application of the sample and elution with a linear gradient of 0.01 M pyridine/acetic acid, pH 5.0, in the mixing chamber and an equal volume of 1.0 M or 0.5 M pyridine–acetic acid, pH 5.0, in the reservoir. Affinity chromatography on lectin–Sepharose columns was done as described (Bhavanandan & Katlic, 1979a). The WGA–Sepharose columns used had about 2 mg of lectin per mL of Sepharose. The recovery of radioactivity and standards from the columns was greater than 80% unless otherwise specified. The distribution of radioactivity in the peaks is reported as percentages of the total recovered.

*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 v/v), (B) 1-butanol/pyridine/water (6:4:3 v/v), (C) pyridine/ethyl acetate/water/acetic acid (5:5:3:1 v/v) with pyridine/ethyl acetate/water (11:40:6 v/v) in the bottom of the chromatography tank, and (D) 1-butanol/acetic acid/1 M $NH_4OH$ (2:3:1 v/v).

Marker neutral sugars, hexosamines, oligosaccharides, and oligosaccharide alcohols were located by the silver nitrate staining procedure (Trevelyan et al., 1950) or by the periodate–benzidine technique (Gordon et al., 1956). Sialic acids were detected by the thiobarbituric acid spray (Warren, 1960) and the *N*-acetylhexosamines and Gal→GalNAc by the Morgan–Elson reagent (Partridge, 1948).

*Determination of [$^3$H]Sialic Acids and [$^3$H]Hexosamines.* [$^3$H]Sialic acid in isotopically labeled glycoproteins was determined by acid hydrolysis (0.1 M $H_2SO_4$, 80 °C, 1 h) or by treatment with *Vibrio cholerae* neuraminidase followed by addition of carrier sialic acid (0.5–1.0 mg) and fractionation on a Bio-Gel P-2 column. Aliquots of the fractions were analyzed for sialic acid and radioactivity. [$^3$H]Hexosamines in the labeled glycoconjugates were estimated after acid hydrolysis (4 M HCl, 100 °C, 8 h). Standard hexosamines and [$^{14}$C]hexosamines were added to the dried hydrolysates, and the mixture was analyzed by a stream-splitting technique on an amino acid analyzer (Bhavanandan & Davidson, 1976) or on a column of AG50W-X8 (H+) according to Gardell (1953). The fractions were analyzed for hexosamine by the ninhydrin or the Elson–Morgan assay (Levvy & McAllan, 1959) and for radioactivity.

*Digestions with Enzymes. Vibrio cholerae* neuraminidase digestion was done in 0.1 M sodium acetate buffer, pH 5.6, containing 1 mM $Ca^{2+}$ with 1.0 unit of enzyme in a total digest volume of 200 μL. Incubations with jack bean or *D. pneumoniae* β-galactosidase were done in 50 mM citrate buffer, pH 4.0, or 50 mM citrate/phosphate buffer, pH 7.0, with 0.2 unit of the enzyme at 37 °C for 24 h in a total of 200 μL. Digestion with *D. pneumoniae* β-hexosaminidase was done in 50 mM citrate/phosphate buffer, pH 5.3, with 0.2 unit of the enzyme. Treatment with endo-α-*N*-acetylgalactosaminidase from *D. pneumoniae* was done in 50 mM Tris/maleate buffer, pH 7.6, at 37 °C for 24 h in a total volume of 50 μL (Umemoto et al., 1977). Digestion with endoglycosidase D or H was done in 50 mM citrate/phosphate buffer (D, pH 6.5; H, pH 5.0) at 37 °C for 20 h (Koide & Muramatsu, 1974). Incubation with *E. Freundii* endo-β-galactosidase was in 0.05 M sodium acetate buffer, pH 5.8, at 37 °C for 24 h (Nakagawa et al., 1980). All digestions were terminated by heating at 100 °C for 2–5 min. The mixtures were centrifuged and the supernatants fractionated on a Bio-Gel P-2 column (0.9 × 70 cm) calibrated with known saccharides. In some experiments, appropriate carrier sugars (GlcNAc, Gal, and AcNeu) were added to the supernatants prior to chromatography.

*Alkaline Borohydride Treatment of Glycopeptides.* Alkaline borohydride treatment of the glycopeptides was done with 1.0 M $NaBH_4$ in 0.1 M NaOH for 72–96 h at 37 °C under nitrogen in sealed tubes in the dark. The reaction mixture was cooled in an ice bath, the excess borohydride destroyed, and the mixture neutralized by careful addition of 1 M acetic acid. The eliminated products were analyzed by chromatography on calibrated columns of Bio-Gel P-4 and P-6.

Other methods used in this study are described in the following paper in this issue (Bhavanandan, 1981).

## Results

Several experiments were carried out on the melanoma cells over a period of about 4 years by using early (<20) and late passage (>100) cells. There was no significant difference between the results from individual experiments; the results presented are representative.

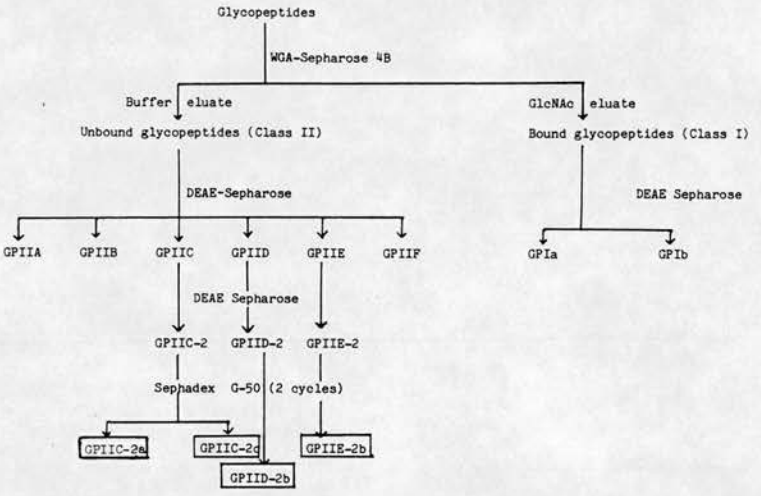
The processing of the labeled glycoconjugates from the cells, spent media, and EGTA supernatants and the cetylpyridinium chloride fractionation are described in the following paper (Bhavanandan, 1981). The glycopeptides were recovered from the cetylpyridinium chloride supernatant. The total radioactivity from [$^3$H]glucosamine incorporated into glycopeptides by the melanoma culture was 93.8 × $10^6$ dpm per $10^7$ cells compared to 57.8 × $10^6$ dpm for $10^7$ cells of the melanocyte culture (see Table I, Bhavanandan, 1981).

*Gel Filtration Analysis of the Glycopeptides.* The mixtures of glycopeptides obtained from melanoma and melanocytes were examined on Sephadex G-50 (not illustrated) and Bio-Gel P-10 (Figure 1) columns. On both columns, the bulk of the melanocyte glycopeptides were included, and only 1–2% eluted in the void volume. In contrast, the melanoma glycopeptides eluting at the void volume of the Bio-Gel P-10 column represented 5 times as much material.

*Affinity Chromatography of the Glycopeptides on a WGA–Sepharose Column.* The results illustrated in Figure 2 show striking differences in the proportions of the glyco-

[1] Abbreviations used: glyceryl-CPG, glyceryl controlled pore glass beads; WGA, wheat germ agglutinin; $RCA_{120}$ and $RCA_{60}$, *Ricinus communis* agglutinins of $M_r$ 120000 and 60000, respectively; class I and class II glycopeptides refer to WGA-binding and WGA-nonbinding glycopeptides, respectively; DEAE, diethylaminoethyl; EGTA [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; AcNeu, *N*-acetylneuraminic acid; Tris, tris(hydroxymethyl)aminomethane.

Scheme I: Fractionation of Glycopeptides Isolated from HM7 Human Melanoma Cells



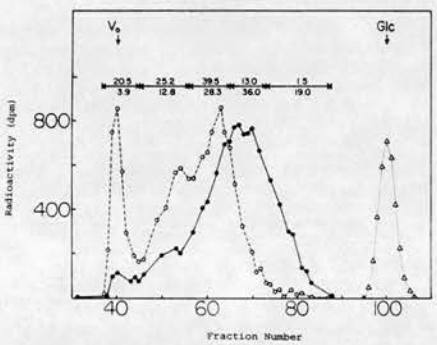




FIGURE 1: Elution profiles of the $^3$H-labeled glycopeptides from human fetal melanocytes and human melanoma cells on Bio-Gel P-10. Human fetal melanocytes labeled with [$^3$H]glucosamine were processed, digested with Pronase, and treated with cetylpyridinium chloride as described in the text. The $^3$H-labeled glycopeptides (●) remaining in the cetylpyridinium chloride supernatant were recovered, mixed with [$^{14}$C]glucose, and chromatographed on a Bio-Gel P-10 column (0.9 × 134 cm). The corresponding $^3$H-labeled glycopeptides isolated from HM7 human melanoma cells (○) were also mixed with [$^{14}$C]glucose and chromatographed on the same column. The column was eluted with 0.1 M pyridine/0.1 M acetic acid (pH 5.0) at 20 mL/h, and 1-mL fractions were collected and analyzed for radioactivity. The results are presented by superimposing the elution patterns, using elution of [$^{14}$C]glucose (△) and the void volume ($V_0$) as references. The percent of radioactivity of the melanoma and melanocyte glycopeptides eluting in the area indicated by the horizontal arrows are given above and below the arrows, respectively.

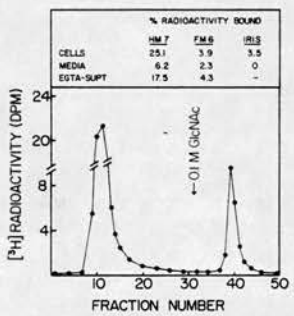



FIGURE 2: Affinity chromatography of glycopeptides on WGA–Sepharose. Glycopeptides isolated from cells, medium, and EGTA supernatant of cell cultures labeled with [$^3$H]glucosamine, as described under Experimental Procedures, were chromatographed on a WGA–Sepharose column (2 × 6.5 cm) containing about 2 mg of WGA covalently bound per mL of gel. The column was eluted with 0.05 M Tris-HCl, pH 8.0, followed by 0.1 M GlcNAc in the same buffer. One-milliliter fractions were collected and analyzed for radioactivity. The results of the elution of melanoma cell-associated glycopeptides are illustrated. The percentage of glycopeptides which specifically bound to WGA when about 50 000 dpm of the glycopeptides from the different cellular compartments of melanoma, melanocytes, and iris were chromatographed is given in the inset. In preparative experiments, the WGA-binding and -nonbinding glycopeptides were recovered by dialysis followed by lyophilization and designated class I and II glycopeptides, respectively.

peptides derived from melanoma, melanocyte, and iris (Banks et al., 1976) cultures specifically binding to WGA. There is a significant reduction of the WGA-binding (class I) glycopeptides in the melanocyte culture and the iris explant when compared to the melanoma culture.

We have previously demonstrated that the binding of sialoglycopeptides to a WGA–Sepharose column depended not only on the number and arrangement of the binding sugar residues (*N*-acetylneuraminic acid or *N*-acetylglucosamine) but also on the density of lectin molecules on the gel beads (Bhavanandan & Katlic, 1979a). Therefore, in the present studies, we have used WGA–Sepharose columns having the same concentration of lectin (about 2 mg/mL). This precaution was important because in subsequent studies we have noticed that on WGA–Sepharose columns having a high density of lectin (10 mg/mL gel), even small serum-type glycopeptides (such as fetuin glycopeptide A) are partially retained (V. P. Bhavanandan, unpublished results).

*Fractionation of the Glycopeptides from Cell, Spent Medium, and EGTA Supernatant (Refer to Scheme I).* The glycopeptides were separated into WGA-binding (class I glycopeptides) and nonbinding (class II glycopeptides) portions by preparative chromatography on WGA–Sepharose (Figure 2).

*Characterization of the Melanoma Class I (WGA-Binding) Glycopeptides.* The melanoma class I glycopeptides were

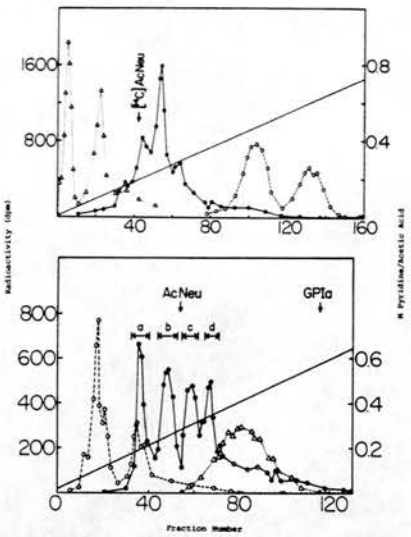



FIGURE 3: Ion-exchange chromatography of glycopeptides on DEAE-Sephacel. (Top) The human melanoma cell-associated glycopeptides [class I (○), class II (●), and asialo class II (△)] isolated from [$^3$H]glucosamine-labeled cultures were each mixed with [$^{14}$C]AcNeu and chromatographed on a DEAE-Sephacel column (0.9 × 40 cm). The column was eluted with a linear gradient of 0.01–1.0 M pyridine/acetic acid, pH 5.0 (400 mL), at the rate of 20 mL/h. Fractions of 2.0 mL were collected and 1-mL aliquots analyzed for radioactivity. The results are presented by superimposing the elution patterns, using elution of [$^{14}$C]AcNeu as an internal reference. (Bottom) Elution profiles of the human fetal melanocyte cell-associated glycopeptides [class I (△), class II (●), and asialo class II (○)].

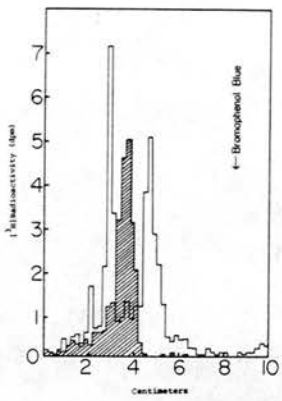



FIGURE 4: Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of human melanoma glycopeptide Ia and glycophorin. The $^3$H-labeled glycopeptide Ia (shaded area) isolated from human melanoma cells and standard [[$^3$H]AcNeu]glycophorin (unshaded area) were electrophoresed on 6% polyacrylamide gel in the presence of sodium dodecyl sulfate for 3 h at 70 V as described by Weber & Osborn (1969). The gels were sliced into 2-mm segments and solubilized by incubation in glass vials with 0.2 mL of 60% perchloric acid and 0.4 mL of 30% hydrogen peroxide at 60 °C for 4 h. Scintillation counting fluid was added to the solubilized gel, and the radioactivity was determined. The results are superimposed by using the migration position of bromophenol blue as reference. Glycophorin yields two bands corresponding to the monomer ($M_r$ 31 000) and dimer ($M_r$ 62 000).

separated into two fractions, GPIa and GPIb, by chromatography on DEAE-Sepharose CL-6B (Figure 3, top). The latter fractions were rechromatographed on a DEAE-Sephacel column and yielded single peaks, as did chromatography on a glyceryl-CPG column. Both these fractions were excluded from Sephadex G-50 and Bio-Gel P-10, but on a Sephacryl S-200 column, these glycopeptides apparently interacted irreversibly since greater than 90% of the radioactivity was lost. Affinity chromatography of GPIa or GPIb on immobilized lectin columns resulted in either binding (WGA) or no binding (concanavalin A, $RCA_{120}$, $RCA_{60}$, and soybean agglutinin) of the entire fractions. Polyacrylamide gel electrophoresis of GPIa and GPIb in 6% gels in the presence of sodium dodecyl sulfate gave single bands. The result obtained with GPIa is illustrated (Figure 4).

*Characterization of the WGA-Binding Glycopeptides GPIa and GPIb.* The distribution of $^3$H radioactivity in sialic acid and hexosamines in the class I glycopeptides is given in Table I. The results of further experiments on the GPIa and GPIb obtained from cells are presented below; analogous studies were also carried out on the fractions isolated from spent medium and EGTA supernatant with substantially similar results.

The class I asialoglycopeptides (mixture of asialo-GPIa and -GPIb) prepared by either mild acid hydrolysis or treatment with neuraminidase did not bind (<5%) to WGA–Sepharose, confirming the role of sialic acid in the interaction with WGA (Bhavanandan et al., 1977; Bhavanandan & Katlic, 1979a,b). The asialoglycopeptides bound to both $RCA_{120}$ and $RCA_{60}$ columns, 79% and 78%, respectively, and could be eluted with 0.1 M lactose. Asialo-GPIa and -GPIb were also separately tested on the $RCA_{60}$ column, and 100% and 83% of the radioactivities were specifically bound, respectively. These results

Table I: Percent Distribution of $^3$H Activity in Sialic Acid and Hexosamines in the Melanoma Class I (WGA-Binding) Sialoglycopeptides[a]

| glycopeptides | sialic acid: acid hydrolysis | sialic acid: neuraminidase | $GlcNH_2$ | $GalNH_2$ |
|---|---|---|---|---|
| cell | | | | |
| class I | 28.4 | 28.0* | 35.1† | 36.5† |
| GPIa | 26.0 | 26.0* | 45.1† | 28.9† |
| GPIb | 40.0 | 42.0* | 10.2† | 49.8† |
| spent medium | | | | |
| class I | 27.4 | ND | 30.5 | 42.1 |
| EGTA supernatant | | | | |
| class I | 28.2 | ND | 33.7 | 38.1 |
| GPIa | 26.5 | ND | 41.9 | 31.6 |
| GPIb | 44.4 | ND | 8.9 | 46.7 |

[a] The sialoglycopeptides were treated with acid or neuraminidase and chromatographed on Bio-Gel P-2 (see Methods for details), and the percent of radioactivity coeluting with carrier sialic acid was estimated. Sialic acids from experiments marked with an asterisk were recovered and identified as *N*-acetylneuraminic acid by paper chromatography in solvent system A. Percent of radioactivity in the hexosamines was determined by hydrolyzing (4 M HCl, 100 °C, 8 h) a separate aliquot of the glycopeptide followed by analysis on a column of AG50W ($H^+$) (samples marked †) or by stream-splitting technique on an amino acid analyzer (details and references are given under Methods). Total radioactivity refers to the sum in sialic acid (acid hydrolysate), $GlcNH_2$, and $GalNH_2$. ND = not determined.

indicated the presence of β-galactosyl or β-*N*-acetylgalactosaminyl nonreducing terminals in these asialoglycopeptides (Irimura et al., 1975); however, heterogeneity with respect to the distribution of oligosaccharides on the peptide may be present (V. P. Bhavanandan, unpublished results).

Treatment of the asialo-GPIa and -GPIb with endo-α-*N*-acetylgalactosaminidase released 25% and 58% radioactivity, respectively, in a product which coeluted on Bio-Gel P-2 columns with Gal1→3GalNAc prepared by treatment of fetuin

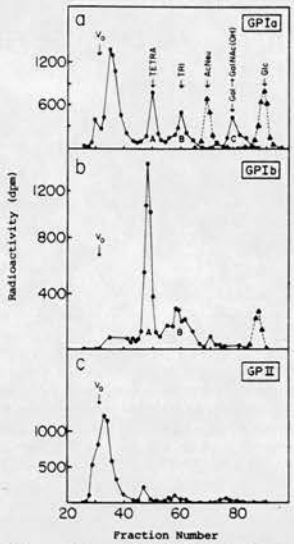



FIGURE 5: Elution profiles on Bio-Gel P-6 of human melanoma glycopeptides after treatment with alkaline borohydride. The human melanoma cell-associated glycopeptides, GPIa (panel a), GPIb (panel b), and GPII, i.e., Class II (panel c), were treated with alkaline borohydride and chromatographed on a Bio-Gel P-6 column (0.9 × 105 cm) as described under Methods. The column was eluted with 0.1 M pyridine/0.1 M acetic acid, 1-mL fractions were collected, and aliquots were analyzed for radioactivity. The elution profiles of internal standards glucose (▲) and *N*-acetylneuraminic acid (▲) and the peak elution positions of blue dextran ($V_0$), AcNeu→Gal→(AcNeu)→-GalNAc(OH) (tetra), AcNeu→Gal→GalNAc(OH) (tri), and Gal→GalNAc(OH) are also indicated.

asialoglycopeptides (Umemoto et al., 1977) with the same enzyme. The product from asialo-GPIa and -GPIb was isolated by gel filtration on a Bio-Gel P-2 column and desalted by passage through mixed-bed ion-exchange resin. Paper chromatography in solvent system A showed that it had a mobility identical with that of reference Gal1→3GalNAc. The identity of the disaccharide was further confirmed by treatment with $NaBH_4$ followed by acid hydrolysis, after which the only labeled component detected on the amino acid analyzer was [$^3$H]galactosaminitol (Bella & Kim, 1970). The sialo- and the asialoglycopeptides Ia and Ib were not degraded by endo-$\beta$-galactosidase from *E. Freundii*.

Alkaline borohydride treatment of GPIa and GPIb followed by gel filtration on a Bio-Gel P-4 or P-6 column indicated that 46% and 88% of the radioactivities, respectively, were released. On both columns, the major products coeluted with [AcNeu→Gal→(AcNeu)→GalNAcOH], [AcNeu→Gal→GalNAcOH], and [Gal→GalNacOH] prepared from fetuin or glycophorin by alkaline borohydride treatment (Spiro & Bhoyroo, 1974) (Figure 5). The major elimination products from GPIa and GPIb were isolated by preparative chromatography on Bio-Gel P-6, repeatedly evaporated with methanol to remove borate (when present), and examined by paper chromatography. In solvent systems A and C, they comigrated with the tetra-, tri-, and disaccharide alcohols isolated from fetuin (Figure 6). Acid hydrolysis of the total $\beta$-eliminated oligosaccharides from GPIb followed by stream-split analysis on the amino acid analyzer employing the citrate–borate buffer system (Bella & Kim, 1970) gave only [$^3$H]galactosaminitol, with no detectable [$^3$H]glucosamine or [$^3$H]galactosamine. Mild acid hydrolysis (0.1 N $H_2SO_4$, 80 °C, 1 h) of the tetrasaccharide followed by chromatography on a Bio-Gel P-6 column gave two sharp peaks coincident with reference AcNeu and Gal1→3GalNAcOH; the radioactivity was distributed equally between the two peaks. The alkaline borohydride resistant portions of GPIa and GPIb (55% and 12%, respectively) eluting prior to the tetrasaccharide peak on the Bio-Gel P-6 column (Figure 5a,b) were also recovered and examined. Only minor portions (14% and 11%) of these fractions were retained on the WGA–Sepharose 4B column. [$^3$H]Glucosamine accounted for greater than 90% of the radioactivity detectable after total acid hydrolysis, in both cases.

Retreatment of GPIa and GPIb with Pronase did not alter their elution profile on the glyceryl-CPG or Sephadex G-50 columns.

*Characterization of the Melanocyte WGA-Binding Glycopeptides.* Ion-exchange chromatography on a DEAE-Sephacel column using a 0.01–1.0 M pyridine/acetic acid gradient gave one broad peak which eluted after the WGA-nonbinding (class II) glycopeptides and before the human melanoma class I glycopeptides Ia (Figure 3, bottom). The percent distribution of radioactivity in sialic acid and hexosamines (Table II) resembles that of melanoma Ia glycopeptides. Alkaline borohydride treatment of WGA-binding glycopeptides from cells followed by gel filtration on a Bio-Gel P-6 column indicated that 38% of the radioactivity was released as oligosaccharides. The hexosamine in the alkaline borohydride resistant portion was practically all glucosamine.

*Studies on the WGA-Nonbinding (Class II) Glycopeptides.* The distribution of radioactivity in sialic acid and hexosamine

Table II: Percent Distribution of $^3$H Activity in Sialic Acid and Hexosamines in the Glycopeptides Produced by Human Melanocytes in Culture and by Explant Iris Culture[a]

| glycopeptides | sialic acid (acid hydrolysis) | $GlcNH_2$ | $GalNH_2$ |
|---|---|---|---|
| FM6 cell | | | |
| class I | 31.5 | 43.8 | 24.7 |
| class II | 20.0* | 66.0 | 14.0 |
| class IIa | 5.3 | 90.8 | 3.9 |
| class IIb | 10.6 | 82.1 | 7.3 |
| class IIc | 14.3 | 75.2 | 10.5 |
| class IId | 16.1 | 71.1 | 12.8 |
| FM6 spent medium | | | |
| class I | 34.0 | 39.5 | 26.5 |
| class II | 20.9* | 65.6 | 13.5 |
| FM6 EGTA supernatant | | | |
| class II | 21.3 | 78.7 | <1 |
| iris explant cell | 16.0 | 73.1 | 10.9 |
| iris explant medium | 18.6 | 76.5 | 4.9 |

[a] Details are as described in Table I.

Table III: Percent Distribution of $^3$H Activity in Sialic Acids and Hexosamines in the Melanoma Class II (WGA-Nonbinding) Sialoglycopeptides[a]

| glycopeptides | sialic acid: acid hydrolysis | sialic acid: neuraminidase | $GlcNH_2$ | $GalNH_2$ |
|---|---|---|---|---|
| cell | | | | |
| class II | 19.6 | 19.1* | 68.3 | 12.1 |
| GPIIC-2a | 15.5 | 14.0* | 79.7 | 4.8 |
| GPIIC-2c | 21.5 | 20.3* | 78.5 | 0.0 |
| GPIID-2b | 23.1 | 21.7* | 76.9 | 0.0 |
| GPIIE-2b | 23.0 | 20.0* | 73.8 | 3.2 |
| spent medium | | | | |
| class II | 22.9 | ND | 72.5 | 4.6 |
| EGTA supernatant | | | | |
| class II | 23.7 | ND | 63.3 | 13.0 |

[a] Details are as described in Table I.

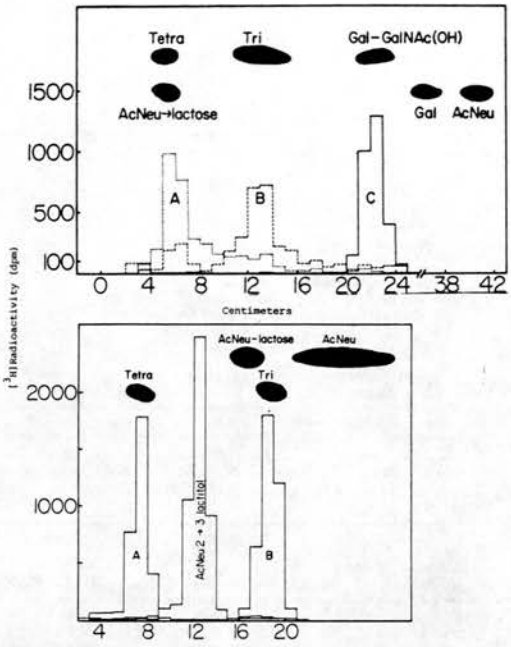



FIGURE 6: Paper chromatography of $\beta$-eliminated oligosaccharides from human melanoma glycopeptides Ia (top panel) and Ib (bottom panel). The $^3$H-labeled oligosaccharides A, B, and C obtained from GPI a (Figure 5, panel a) and A and B from GPIb (Figure 5, panel b) were chromatographed by using solvent systems A and C, respectively. The migration positions of standard sugars detected as described under Methods are indicated. Tetra and Tri refer to the tetra- and trisaccharides described in Figure 5.

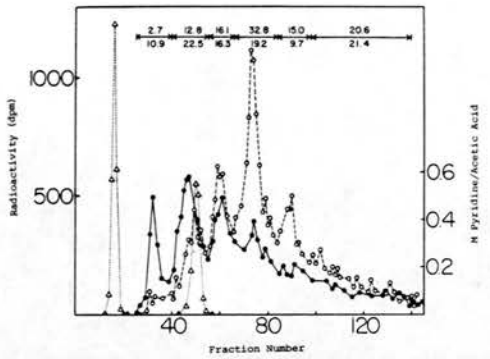



FIGURE 7: Elution profiles of the $^3$H-labeled glycopeptides from human fetal melanocytes and human melanoma cells on DEAE-Sephacel. The WGA-nonbinding (class II) glycopeptides isolated from [$^3$H]-glucosamine-labeled human fetal melanocytes (●) and human melanoma (○) were separately mixed with [$^{14}$C]glucose and [$^{14}$C]AcNeu (△) and chromatographed on a DEAE-Sephacel column (1.5 × 22 cm). The column was eluted with a linear gradient of 0.01–0.5 M pyridine/acetic acid, pH 5.0 (300 mL), at 15 mL/h. Fractions of 2.1 mL were collected and 1-mL aliquots analyzed for radioactivity. The results are presented by superimposing the elution profiles. The present of radioactivity of the melanoma and melanocyte glycopeptides eluting in the area indicated by the horizontal arrows are given above and below the arrows, respectively.

in the class II glycopeptides is given in Tables II and III.

The elution profiles of the WGA-nonbinding glycopeptides obtained from melanoma cells and melanocytes on an ion-exchange column is illustrated in Figure 7. There is a higher proportion of the less anionic glycopeptides in the melanocyte-derived glycoconjugates. For example, 49.7% of the $^3$H-labeled glycopeptides from melanocytes elutes in fractions 25–66 as opposed to only 31.6% in the case of the melanoma-derived glycopeptides. Gel filtration on glyceryl-CPG, Sephadex G-50, and Bio-Gel P-10 indicated that these glycopeptides are heterogeneous and of lower molecular weight when compared to the class I glycopeptides. The melanoma cells, however, contained a higher proportion of the larger glycopeptides than did the melanocytes (cf. Figure 1).

The size of the class II glycopeptides was reduced on desialylation (about 80% of radioactivity was now dialyzable), and the asialoglycopeptides were still highly heterogeneous. It was therefore apparent that the heterogeneity of the class II glycopeptides is not solely due to differences in the degree of sialylation, even though this may be a factor. This was further illustrated by chromatography of the untreated and desialylated glycopeptides on a DEAE-Sepharose column (Figure 3, top).

*Characterization of the Melanoma Class II Glycopeptides.* The melanoma sialo- and asialoglycopeptides on treatment with endo-$\alpha$-*N*-acetylgalactosaminidase or with endo-$\beta$-galactosidase released insignificant quantities of radioactivity. The results of alkaline borohydride treatment of the class II glycopeptides from melanoma cells followed by gel filtration is illustrated in Figure 5c. About 5% and 4% of the radioactivities were in the released tetra- and trisaccharides, respectively.

Attempts to fractionate the class II glycopeptides on WGA–, Con A–, $RCA_{120}$–, and $RCA_{60}$–Sepharose columns were unsuccessful since these glycopeptides were not retained on these columns. The asialoglycopeptides also did not interact with either WGA– or Con A–Sepharose but were partially (77% and 24%) retained on $RCA_{120}$– and $RCA_{60}$–Sepharose columns. On a $RCA_{120}$–Sepharose column, class II asialoglycopeptides separated into three categories: not retained (excluded from the column), 12%; retarded but eluted with buffer, 76%; retained and eluted with 0.1 M lactose, 12%.

*Fractionation of the Melanoma Class II Glycopeptides (Refer to Scheme I).* The class II glycopeptides from melanoma cells were fractionated on a DEAE-Sepharose column into six fractions (GPIIA–F) by using a 0.01–1.0 M pyridine/acetic acid, pH 5.0, gradient. The three major fractions (GPIIC, GPIID, and GPIIE) were separately rechromatographed on DEAE-Sepharose by using a 0–0.5 M pyridine/acetic acid gradient, and the material eluting in sharp peak areas GPIIC-2, GPIID-2, and GPIIE-2 was recovered (Figure 8) and chromatographed on a column (0.9 × 140 cm) of Sephadex G-50 (fine). Fraction GPIIC-2 yielded two peaks and fractions GPIID-2 and GPIIE-2 one major peak each. The material in the major peaks in each case was rechromatographed on the Sephadex G-50 column, recovered, and designated GPIIC-2a, GPIIC-2c, GPIID-2b, and GPIIE-2b. These glycopeptides represented 4.0%, 5.4%, 10.5%, and 3.3% of the total radioactivity in the glycopeptides (cetylpyridinium chloride supernatant).

*Partial Characterization of GPIIC-2a, GPIIC-2c, GPIID-2b, and GPIIE-2b.* The distribution of $^3$H counts in sialic acid and hexosamine in these glycopeptides is given in Table III. All four glycopeptides had a lower proportion of radioactivity in sialic acid and a much higher proportion in glucosamine as compared to GPIa and GPIb. This indicated that these may contain N-glycosidic linkages between GlcNAc and asparagine.

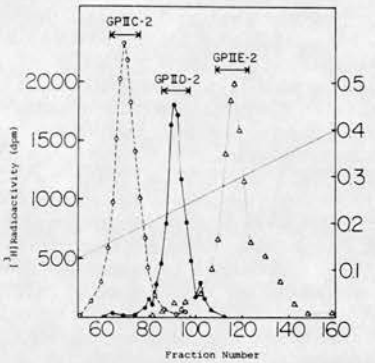



FIGURE 8: Fractionation of $^3$H-labeled class II glycopeptides from human melanoma cells on DEAE-Sepharose. The human melanoma class II glycopeptides isolated from [$^3$H]glucosamine-labeled cells were chromatographed on a DEAE-Sepharose column (1.5 × 25 cm). The column was eluted with a linear gradient of 0.01–1.0 M pyridine/acetic acid, pH 5.0. The total volume of the gradient was 400 mL, and the rate of elution was about 20 mL/h. Fractions of 2 mL were collected, and aliquots of 25 μL were analyzed for radioactivity. The glycopeptides eluting in fractions 42–51, 52–62, and 63–74 were recovered by lyophilization and designated GPIIC, GPIID, and GPIIE. They were then individually rechromatographed on the same DEAE-Sepharose column by using a 0–0.5 M pyridine/acetic acid gradient. In each case, the material eluting in the area indicated by horizontal arrows (GPIIC-2, GPIID-2, and GPIIE-2) was recovered by lyophilization.

Asialo-GPIIC-2c and -GPIID-2b, prepared by treatment with *V. cholerae* neuraminidase followed by chromatography on Bio-Gel P-2, interacted specifically with $RCA_{120}$–Sepharose but not with Con A–Sepharose. Treatment of the asialo-GPIIC-2c and -GPIID-2b with *D. pneumoniae* β-galactosidase and β-*N*-acetylhexosaminidase released a product containing 45% and 55% of the radioactivity, respectively. This product eluting in the same position as GlcNAc on a Bio-Gel P-2 column was isolated and desalted by passage through Dowex AG50 (H+) and Dowex AG1 (formate) columns. On paper chromatography using solvent system B, it was indistinguishable from standard GlcNAc. In control experiments, treatment of the asialoglycopeptides with either enzyme alone failed to release any labeled products.

The portion of asialo-GPIIC-2c undigested by the exoglycosidases eluting at the void volume of the Bio-Gel P-2 column was recovered by lyophilization. It was not retained on a Con A–Sepharose column and was resistant to degradation by endo-*N*-acetylglucosaminidase H. However, on treatment with endo-*N*-acetylglucosaminidase D followed by chromatography on a Bio-Gel P-4 column, 32% of the radioactivity eluted in the position of reference GlcNAc→Asn. Hydrazinolysis of GPIIC-2c and GPIID-2b followed by gel filtration of a Bio-Gel P-6 column gave included peaks, indicating that these glycopeptides had oligosaccharides linked N-glycosidically to the peptide.

*Fractionation of the Melanocyte Class II Glycopeptides.* The cell-associated class II sialoglycopeptides were preparatively fractionated on the DEAE-Sepharose column into four fractions (a–d). The distribution of radioactivity in the peaks marked a, b, c, and d in Figure 3 (bottom) was 23%, 33%, 23%, and 21%. The fractions were rechromagraphed on a DEAE-Sephacel column using 0–0.5 M pyridine/acetic acid gradient, and the material eluting in sharp peaks was recovered and designated class IIa, class IIb, and class IIc, and class IId. The distribution of $^3$H activity in sialic acid and the significant quantities of galactosamine in these glycopeptides compared to the human melanoma class II glycopeptides (Table III) is of interest. The results suggest that the major portion of the carbohydrate in these glycopeptides is of the N-glycosidically linked type. Insufficient material was available for further purification and characterization of these four subfractions.

*Characteristics of Glycopeptides from Human Embryonic Iris.* Chromatography on Sephadex G-50 of the glycopeptides from both tissue and medium gave patterns very similar to that of the asialoglycopeptides isolated from cultured melanocytes. On gel filtration on a calibrated CPG column, the iris glycopeptides eluted as a broad peak, with a maximum two fractions before glucuronic acid. The elution pattern of the iris glycopeptides on a DEAE-Sepharose column was unusual and markedly different from those of the melanocyte and melanoma glycopeptides. A significant portion (27%) of the iris glycopeptides eluted in a sharp peak very early; these are probably uncharged (nonsialylated) or low sialic acid glycopeptides. The overall reduction in sialylation in the iris glycopeptides is also reflected by the low percent of radioactivity in the sialic acid, 16.0% and 18.6%, for the tissue and medium derived glycopeptides, respectively. Alkaline borohydride treatment of these iris medium glycopeptides followed by chromatography on Bio-Gel P-6 showed that about 12% of the radioactivity was released as tetra- and trisaccharides. This is in agreement with the presence of 11% and 5% [$^3$H]-galactosamine in the iris tissue and medium glycopeptide, respectively. The elution pattern of the elimination products on the Bio-Gel P-6 column was very similar to that obtained for melanocyte glycopeptides after the same treatment. These O-glycosidically linked oligosaccharides are apparently not present as clusters and thus fail to bind to the WGA–Sepharose.

## Discussion

The WGA-binding glycopeptide, GPIb, had a mobility on a glyceryl-CPG column identical with that of chondroitin 4-sulfate from cartilage and of mouse melanoma class I glycopeptides (Bhavanandan & Davidson, 1976), indicating an apparent molecular weight in the range of 12000–15000, whereas glycopeptide GPIa had an apparent molecular weight of ~8000–10000. The reason for the anomalous behavior of GPIa on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 4) is not entirely clear but is probably dependent on higher carbohydrate content of GPIa (expected to be greater than 90%) compared to that of glycophorin (55%). The high sialic acid content is likely to be the primary determinant of the ion-exchange chromatographic behavior of class I glycopeptides Ia and Ib (Figure 3, top) even though factors such as amino acid composition and size are likely to contribute.

Chromatographic and enzymatic data suggest the presence of the sequence Gal1→3[$^3$H]GalNAc in asialo-GPIa and -GPIb. GPIb has a large portion of tetra- (51%) and trisaccharides (31%) linked O-glycosidically in clusters to serine or threonine, as suggested by affinity chromatography on WGA–Sepharose (Bhavanandan & Katlic, 1979a) and resistance to degradation by Pronase. Thus GPIb has a structure very similar to that suggested for the mucin-type (class I) sialoglycopeptides obtained from mouse melanoma (Bhavanandan & Davidson, 1976; Bhavanandan et al., 1977). However, it differs from the mouse melanoma glycopeptide in containing a small proportion of N-glycosidically linked glucosamine-containing oligosaccharides and in not being precipitated by cetylpyridinium chloride. Since neither glycophorin nor glycopeptides derived from it is precipitated to any extent by this cationic detergent, the class I mouse me-

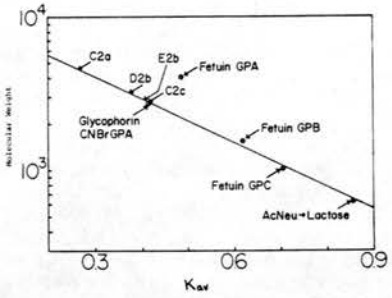



FIGURE 9: Molecular weight estimation of human melanoma glycopeptides IIC-2a, IIC-2c, IID-2b, and IIE-2b. The purified $^3$H-labeled human melanoma glycopeptides IIC-2a, IIC-2c, IID-2b, and IIE-2b were individually mixed with $^{14}$C-labeled fetuin and [$^{14}$C]-glucose and chromatographed on the calibrated Sephadex G-50 column. The $K_{av}$ values are plotted against log molecular weight.

lanoma glycopeptides may contain other anionic groups (sulfate or phosphate) or a closer spacing of the sialic acid residues and therefore a higher charge density. Further work is necessary to resolve this discrepancy.

The higher proportion of glucosamine, the difference in the ratio of O-glycosidically linked oligosaccharides, and the inability to convert GPIa to GPIb by treatment with Pronase excludes the possibility that GPIa is a precursor of GPIb, although both bind to WGA–Sepharose.

The four substantially homogeneous WGA-nonbinding (class II) glycopeptides from melanoma cells have apparent molecular weights of 7000, 3700, 4500, and 3800 based on their mobilities on a Sephadex G-50 column (Figure 9). The anomalous behavior of sialoglycopeptides (Alhadeff, 1978; Santer & Glick, 1979) in gel filtration precludes a more accurate determination of their molecular weights by this technique. Glycopeptides IIC-2c and IID-2b were shown to have Gal→GlcNAc sequences penultimate to sialic acid and *N,N'*-diacetylchitobiosyl linkages to asparagine residues, probably with fucose substitution at the internal GlcNAc residue (Muramatsu et al., 1978). The various class II glycopeptides may differ from each other in monosaccharide composition and the number and completeness of the branches, i.e., bi-, tri-, and tetraantennary structures; differences in the core region are also possible.

N-Glycosidically linked simple (oligomannosyl) type oligosaccharides were not detected in these studies, probably due to the isolation procedure which involved extensive dialysis. In fact, the gel filtration profile on Sephadex G-50 of the total Pronase digest of the cells before and after exhaustive dialysis suggests the loss of low molecular weight fragments. Other glycopeptides which would have been lost by dialysis are those having nonclustered O-glycosidically linked oligosaccharides as are present in fetuin. The failure of endo-$\beta$-galactosidase from *E. Freundii* to degrade GPIa, GPIb, or class II sialo- or asialoglycopeptides indicates the absence of repeating Gal→GlcNAc structures in these fractions (Krusius et al., 1978; Li et al., 1980). The sialic acid and hexosamine compositions of the partially purified melanocyte class II glycopeptides and of iris glycopeptides (Table II) show that the major portion of the carbohydrate in these glycopeptides are N-glycosidically linked *N*-acetyllactosaminyl type, although they differ from the analogous melanoma glycopeptides in having significant quantities of galactosamine.

An increase in the molecular size of N-glycosidic complex glycopeptides from malignant cells (Warren et al., 1978) was also observed in this study. The increased production of these higher molecular weight glycopeptides (referred to as group A by Warren et al., 1978) has been correlated with the active growth state and tumorigenicity of cancer cells (Buck et al., 1971; Glick et al., 1973; Muramatsu et al., 1973; Van Beek et al., 1977). The purification and partial structure of one of these larger glycopeptides isolated from Rous sarcoma virus transformed baby hamster kidney cells were recently reported (Glick, 1979; Santer & Glick, 1979).

Another difference noted in our studies was a striking increase in the WGA-binding glycopeptides in the melanoma culture compared to the melanocyte culture (Figures 1 and 2). Similar increases in the production of WGA-binding glycopeptides by B16 mouse melanoma cells (Satoh et al., 1974; Bhavanandan & Davidson, 1976; Bhavanandan et al., 1977) and by two mammary cancer cells (Chandrasekaran & Davidson, 1979) compared to their normal counterparts have also been noted. The relevance of these alterations to malignancy is not clear, but both alterations noted indicate a higher degree of glycosylation (specifically sialylation) in cancer cells. This is in agreement with the overall increase in the negative charge of the cancer cell as detected by whole cell electrophoresis (Abercrombie & Ambrose, 1962) and with the increase in sialyltransferase activities of cancer cells (Warren et al., 1972; Bosmann & Hall, 1974; Bernacki & Kim, 1977). Further, studies with malignant cells in vivo have consistently shown that plasma membrane sialic acid is elevated relative to control cells (Mabry & Carubelli, 1972; Bryant et al., 1974; Bosmann et al., 1974; Dristrian et al., 1977). This increased cell surface sialic acid has been implicated in reducing the immunogenicity of neoplastic cells by masking antigenic determinants.

Since the melanocytes were of embryonic origin, the differences noted in our studies may not be entirely related to the malignant state of the melanoma cells. It is also difficult to draw conclusions regarding the changes in the individual glycoconjugates based solely on the results of these studies. Recently, we succeeded in purifying and partially characterizing a mucin-type glycoprotein from HM7 human melanoma cells (Umemoto et al., 1981).

Acknowledgments

We wish to thank Dr. Y.-T. Li, Tulane University, for providing the exo- and endo-$\beta$-galactosidases.

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*Biochimica et Biophysica Acta*, 646 (1981) 402–410
Elsevier/North-Holland Biomedical Press

BBA 79376

# ISOLATION AND PARTIAL CHARACTERIZATION OF A MUCIN-TYPE GLYCOPROTEIN FROM PLASMA MEMBRANES OF HUMAN MELANOMA CELLS

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(Received March 9th, 1981)

*Key words: Glycoprotein isolation; Plasma membrane; Mucin; (Human melanoma cell)*

**Plasma membranes were isolated from HM7 melanoma cells grown in the presence of [$^3$H]glucosamine and $Na_2^{35}SO_4$ or [$^3$H]mannose and [$^{14}$C]glucosamine. The labeled glycoconjugates were solubilized with 0.6 M lithium diiodosalicylate/0.5% Triton X-100. Fractionation of glycoconjugates by repeated chromatography on columns of Sepharose CL-6B and DEAE-Sepharose and by affinity chromatography on WGA-Sepharose yielded three radiochemically homogenous glycoproteins. One of these having an apparent molecular weight of 100 000 was found to contain clusters of $(AcNeu)_{1\text{ or }2} \rightarrow [Gal \rightarrow GalNAc]$ linked *O*-glycosidically to the protein. One other glycoprotein contained both *O*-glycosidically and *N*-glycosidically-linked oligosaccharides, and the third contained only *N*-glycosidically-linked carbohydrates. Preliminary results indicate that the 100 000 molecular weight mucin-type glycoprotein is present in significantly reduced quantities in cultured human fetal uveal melanocytes. Further, the bulk of the glycoproteins from the melanocytes were of lower molecular size compared to those from the melanoma cells.**

## Introduction

We recently described the characterization of glycopeptides derived from HM7 human melanoma cells and from human fetal uveal melanocytes [1]. The patterns of glycopeptides obtained from these two sources were strikingly different. The melanoma cells produced increased quantities of the two wheat germ agglutinin (WGA)-binding mucin-type glycopeptides. These glycopeptides were shown to have highly sialylated oligosaccharides linked *O*-glycosidically to protein, in clusters. On the other hand, for the WGA-nonbinding glycopeptides, there was a significantly higher proportion of the smaller molecular size less anionic species derived from melanocytes compared to those from the melanoma cells. Similar increases in the size of the *N*-glycosidic glycopeptides from cancer cells and tumors have been reported by several workers (see review by Warren et al. [2]).

In the present study, we have succeeded in isolating three glycoproteins from plasma membranes of HM7 human melanoma cells. The partial characterization of an acidic mucin-type glycoprotein is discussed. A preliminary report has been presented [3].

## Materials and Methods

### *Materials*

The HM7 human melanoma cells [1] and cultured FM13 human fetal uveal melanocytes [4,5] described previously were used in these studies. Pronase CB and *Vibrio cholerae* neuraminidase (EC 3.2.1.18) were

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Abbreviations: WGA, wheat germ agglutinin; AcNeu, *N*-acetylneuraminic acid; GalNAc(OH), *N*-acetylgalactosaminitol.

purchased from Calbiochem (La Jolla, CA). Sepharose CL-6B, DEAE-Sepharose CL-6B and Sephadex G-50 were obtained from Pharmacia (Piscataway, NJ). Endo-α-*N*-acetylgalactosaminidase (EC 3.2.1.97) from *Diplococcus pneumoniae* culture filtrates was prepared in our laboratory [6]. Wheat germ agglutinin was isolated and conjugated as described [7]. D-[6-$^3$H]glucosamine hydrochloride; [1-$^{14}$C]glucosamine hydrochloride; $Na_2^{35}SO_4$ and D-[1-$^3$H]mannose were obtained from New England Nuclear (Boston, MA). Lithium 3,5-diiodosalicylate was purchased from Eastman Kodak Co. (Rochester, NY). The oligosaccharides, $(AcNeu)_{0-2} \rightarrow [Gal \rightarrow GalNAcOH]$, were isolated from fetuin by treatment with alkaline borohydride as described [8].

*Methods*

*Column chromatography*. Sepharose CL-6B columns were equilibrated and eluted with Tris-HCl buffer, pH 8.0 containing 0.1% Triton X-100 and 0.1 mM toluenesulfonyl fluoride; Sephadex G-50, and Bio-Gel P-2 and P-6 columns were equilibrated and eluted with 0.1 M pyridine acetate, pH 5.0. DEAE-Sepharose CL-6B columns were packed according to the manufacturer's instructions and regenerated to the chloride form. After application of the sample, the columns were eluted with linear gradients of LiCl in Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 and 0.1 mM toluenesulfonyl fluoride. Affinity chromatography on a WGA-Sepharose column, containing about 5 mg lectin per ml Sepharose, was done as described [7]. The recovery of the radioactivity from the columns was better than 80%.

*Digestions with enzymes*. Pronase digestion was performed for 96 h at 37°C in 50 mM Tris-HCl buffer, pH 8.0 containing 10 mM $Ca^{2+}$ in the presence of toluene. Pronase dissolved in buffer was added at 0, 24, 48 and 72 h. The enzyme preparation used was free of contaminating exoglycosidases and endo-*N*-acetylhexosaminidases when assayed with *p*-nitrophenyl glycosides and [$^3$H]acetylovalbumin glycopeptides, respectively [6]. Treatment with endo-α-*N*-acetylgalactosaminidase from *D. pneumoniae* was done in 50 mM Tris-maleate buffer, pH 7.6 at 37°C for 24 h in a total volume of 50 μl. *V. cholerae* Neuraminidase digestion was done in 0.1 M sodium acetate buffer, pH 5.6, containing 1 mM $CaCl_2$ using 0.5 unit of enzyme, at 37°C for 24 h. All digestions were terminated by heating at 100°C for 2–5 min.

Alkaline borohydride treatment of the glycopeptides was done with 1.0 M $NaBH_4$ in 0.1 M NaOH for 72–96 h at 37°C under nitrogen in sealed tubes in the dark. The reaction mixture was cooled in an ice bath, the excess borohydride destroyed and the mixture neutralized by careful addition of 1 M acetic acid.

All dialysis and concentrations were carried out at 4°C in Amicon Ultrafiltration units using PM-10 membranes.

Liquid scintillation counting was performed on an Intertechnique Model SL-36 or Model SL-4000 spectrometer equipped with dpm calculating modules. Usually 0.3- or 1.0-ml aqueous samples were mixed with 3 or 10 ml of counting liquid in mini- or regular plastic vials, respectively. Using the external standard method, quenching was not detectable with any of the buffers. Radioactivity on paper chromatogram strips was estimated by extracting cut pieces with 1 ml water in counting vials prior to addition of counting liquid.

*Cell cultures*. The conditions of cell culture and of labeling of the complex saccharides produced by the cells have been described [1,5].

*Preparation of plasma membranes*. The cultured cells were treated with 0.02% EGTA in NaCl/$P_i$ and washed twice with 0.15 M NaCl. The cells were suspended in 2 mM $ZnCl_2$, at $2 \cdot 10^8$ cells per 10 ml, and after 15 min at 20°C the suspension was placed in an ice bath [9]. After 5 min, the cells were homogenized in a tight fitting (Type B) Dounce homogenizer until greater than 90% of the cells were broken. The homogenate was centrifuged at $220 \times g$ for 15 min and the pellet was washed once with 10 ml 2 mM $ZnCl_2$.

The membranes were isolated from this pellet by partition in an aqueous two phase polymer system as described by Brunette and Till [10]. Essentially, the pellet was suspended in 10 ml of the top phase, mixed with an equal volume of the bottom phase and centrifuged at $9\,000 \times g$ for 10 min. The supernatant containing the membranes at the interface was transferred to another tube, mixed and re-centrifuged. This process was repeated two or three times. The pellets were combined and used for the isolation of nuclei. The results of the investigation of the nuclear glycoconjugates will be reported elsewhere (Bhava-

nandan, V.P., unpublished data). The material at the interface after the final centrifugation in the two phase system was recovered and subjected to discontinuous sucrose-density centrifugation [11]. The membrane fraction banding between 37% and 41% sucrose was used in our studies.

*Solubilization of plasma membrane glycoconjugates*. The membranes were delipidated by two extractions with chloroform/methanol (2 : 1, v/v) and then suspended in 5 ml of 0.6 M lithium diiodosalicylate and 0.5% Triton X-100 in 0.1 M Tris-HCl buffer, pH 7.5 [12]. The suspension was stirred at 4°C for 2 h and then centrifuged at 11 000 × *g* for 20 min. This process of extraction and centrifugation was repeated two more times, with the final extraction for 4 h. The combined supernatants, containing 90% and 95% of the $^{3}H$- and $^{35}S$-labeled components respectively, were dialysed against 50 mM Tris-HCl buffer, containing 0.1% Triton and 0.1 mM toluenesulfonyl fluoride and concentrated.

*Marker enzyme assays*. 5′-Nucleotidase was assayed by the method of Dewald and Touster [13] and succinate dehydrogenase as described by Pennington [14]. ($Na^+$, $K^+$)-activated ATPase and NADPH-diaphorase were determined by the procedures of Wallach and Kamat [15]. Protein was determined according to Lowry et al. [16] with crystalline bovine serum albumin as standard. For electron microscopic examination the membrane fractions were fixed in 2% glutaraldehyde and post fixed with osmium tetroxide. Sections of the embedded material were examined in an RCA model EMU-4 electron microscope. We are indebted to Mr. Barry Hillman for his assistance in this work.

## Results

Plasma membranes were obtained in good yields by homogenization of cells in the presence of Zn ions followed by centrifugation in a two phase aqueous polymer system and sucrose density centrifugation. The purity of the membrane preparation was established by assay of marker enzymes and by electron microscopic examination. The membranes obtained from cells metabolically labeled with [$^{3}H$]glucosamine and $Na_2^{35}SO_4$ were treated with buffer con-

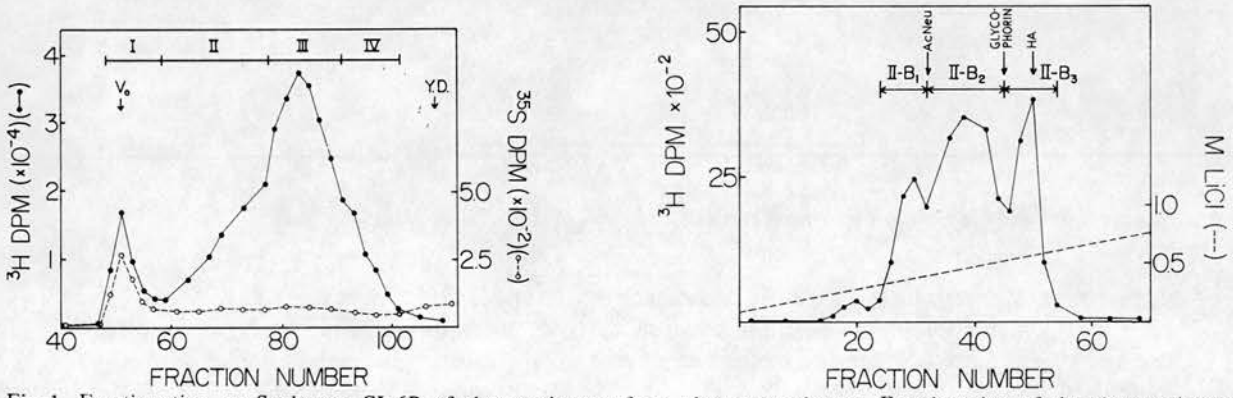
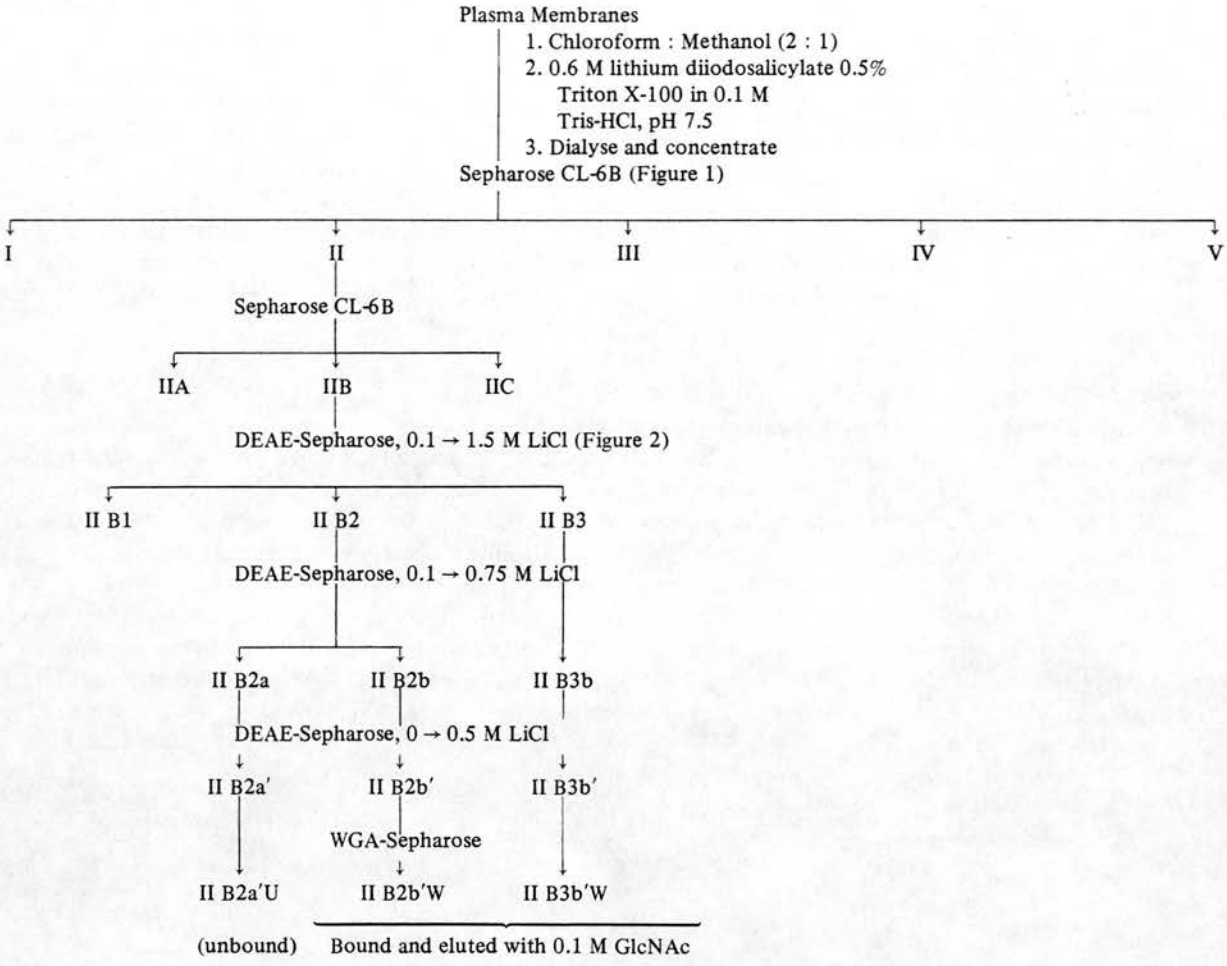



Fig. 1. Fractionation on Sepharose CL-6B of glycoconjugates from plasma membranes. Fractionation of the glycoconjugates extracted by 0.6 M lithium diiodosalicylate/0.5% Triton X-100 from plasma membranes of HM7 human melanoma cells metabolically labelled with [$^{3}H$]glucosamine and $Na_2^{35}SO_4$. The extracts were dialysed against 50 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 and 0.1 mM toluenesulfonyl fluoride, concentrated and chromatographed in the same buffer on a column of Sepharose CL-6B (1.5 × 100 cm). Fractions (1.2 ml) were collected and aliquots analyzed for radioactivity. Fractions were combined as indicated and concentrated to yield the numbered peaks. Peak elution positions of blue dextran ($V_0$) and yellow dextran (Y.D.) are indicated by arrows.

Fig. 2. Fractionation of glycoprotein fraction IIB on DEAE-Sepharose. The glycoprotein Fraction IIB from the second gel filtration on Sepharose CL-6B was chromatographed on a DEAE-Sepharose column (1.5 × 18 cm). The column was eluted with a linear gradient of 0.1 to 1.5 M LiCl in 50 MM Tris-HCl, pH 8.0, containing 0.1% Triton X-100 and 0.1 mM toluenesulfonyl fluoride. Fractions of 1 ml were collected and aliquots analyzed for radioactivity. The three fractions indicated by horizontal arrows were recovered by dialysis and concentration. The peak elution positions of AcNeu, glycophorin and hyaluronic acid (HA) are indicated.




Scheme 1: Isolation of Glycoproteins from Plasma Membranes of HM7 Human Melanoma Cells.

taining various detergents, singly or in combinations [17]. The combination of 0.6 M lithium diiodosalicylate and 0.5% Triton was found to be the best since it solubilized about 90 and 95% of the $^{3}H$- and $^{35}S$-labeled components, respectively.

*Fractionation of the solubilized glycoconjugates*

The fractionation of the solubilized plasma membrane glycoconjugates on a column of Sepharose CL-6B is illustrated in Fig. 1. The five peaks obtained were designated I, II, III, etc. The material from each peak was digested with pronase and the resultant glycopeptides fractionated by gel filtration on a Sephadex G-50 column into peak A (excluded) and peaks B and C (included). Hexosamine analysis, affinity chromatography on WGA-Sepharose and treatment with alkaline borohydride of the three glycopeptide peaks revealed that peak A was enriched in the Class I (mucin-type) glycopeptides reported previously [1]. The Sepharose CL-6B fractions I and II were both rich in the peak A glycopeptides but fraction II, containing 22% of the total $^{3}H$-radioactivity, was chosen for further purification since it was low in $^{35}SO_4$ and thus in sulfated glycosaminoglycans. Rechromatography of II on Sepharose CL-6B gave a rather broad peak and the material in fractions 60–77 was recovered to yield IIB. Chromatography of IIB on a DEAE-Sepharose column using a 0.1 → 1.5 M LiCl gradient (Fig. 2) resulted in three peaks (IIB1, IIB2 and IIB3). Fractions IIB2 and IIB3 were sepa-

rately rechromatographed on the DEAE-Sepharose column but using a gradient of 0.1→0.75 M LiCl for elution, to yield three fractions IIB2a, IIB2b and IIB3b. Each of these fractions was chromatographed once more on DEAE-Sepharose columns using 0 → 0.5 M LiCl gradients and the material eluting in the sharp symmetrical areas of the peaks recovered. Additional purification of the peak materials was achieved by affinity chromatography on a WGA-Sepharose column. The major portion (84%) of IIB2a′ which passed through the column was recovered and designated IIB2a′ U. In the case of IIB2b′ and IIB3b′, 88 and 99%, respectively, bound to the WGA-Sepharose and could be eluted with 0.1 M *n*-acetylglucosamine. The materials recovered by dialysis and concentration were designated IIB2b′W and IIB3b′W. The three purified glycoproteins contained about 7% of the total $^3$H-radioactivity in the glycoconjugates (lithium diiodosalicylate extract); see Scheme I.

*Characterization of the glycoprotein fractions*

Polyacrylamide gel electrophoresis [18] of the three purified glycoproteins in 6% gel in the presence of sodium dodecyl sulfate gave single bands. Gel filtration of the glycoproteins on a Sepharose CL-6B column using buffer containing 0.1% Triton X-100 gave single homogenous peaks in each case. The apparent molecular weight of IIB3b′W was estimated to be about 100 000 based on gel filtration on a column calibrated with glycoprotein standards (Fig. 3).

TABLE I

DISTRIBUTION OF TRITIUM ACTIVITY IN SIALIC ACID AND HEXOSAMINES OF PURIFIED GLYCOPROTEINS

The proportions of sialic acid in three purified glycoproteins were determined by hydrolysis (0.1 N $H_2SO_4$, 80°C, 1 h), neutralization and gel filtration on a BioGel P-2 column. The tritium activity co-eluting with [$^{14}$C]AcNeu internal standard was estimated. Hexosamine was determined on acid hydrolysates (4N HCl, 100°C, 8 h) on an amino acid analyser by a streamsplitting technique (22).

| Glycoprotein | Sialic Acid (%) | $GlcNH_2$ (%) | $GalNH_2$ (%) |
|---|---|---|---|
| II B2a′U | 20.0 | 78.8 | 1.2 |
| II B2b′W | 26.3 | 49.9 | 23.8 |
| II B3b′W | 37.1 | 1.9 | 61.0 |

The distribution of tritium activity in sialic acid and hexosamines in the three glycoproteins is given in Table I.

*Elucidation of the nature of oligosaccharides in IIB3b′W*

The glycoprotein was subjected to alkaline borohydride treatment and chromatographed on a calibrated BioGel P-6 column. The $^3$H-labeled carbohydrate was almost completely (94%) eliminated and about 55 and 29% eluted in the positions of the tetrasaccharide [AcNeu → Gal → (AcNeu) → GalNAc-(OH)] and the trisaccharide [AcNeu → Gal → GalNac-(OH)], respectively (Fig. 4). When the material from these two peaks were isolated, treated with *V. cholerae* neuraminidase and rechromatographed on the

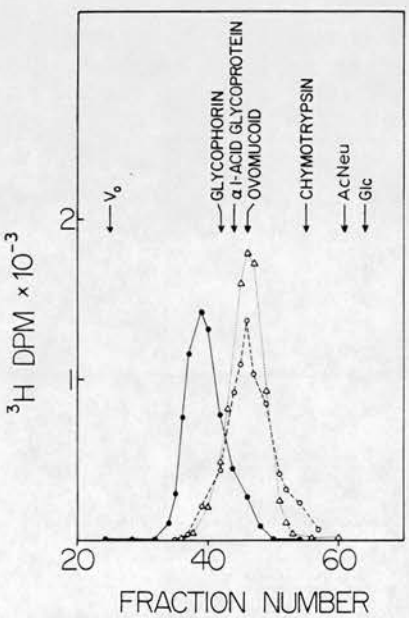



Fig. 3. Gel filtration of IIB3b′W on Sepharose CL-6B before and after pronase digestion. The glycoprotein IIB3b′W before (•——•) or after (○- - - - -○) pronase digestion was mixed with [$^{14}$C]glucose and chromatographed on a Sepharose CL-6B column (1.5 X 35 cm). The column was eluted with 50 mM Tris-HCl, pH 8.0, containing 0.1% Triton X-100 and 0.1 mM toluenesulfonyl fluoride. 1-ml fractions were collected and analyzed for radioactivity. Human melanoma Class I glycopeptide GPIb (△ · · · · · · △) was also similarly chromatographed. The results are presented by superimposing the elution patterns using [$^{14}$C]glucose as an internal reference. The peak elution positions of blue dextran ($V_0$), AcNeu, Glc and several glycoproteins are indicated by arrows.

same column the radioactivity eluted in a single peak coincident with Gal → GalNAc(OH) and AcNeu (Fig. 4). The presence of Gal → [$^3$H]GalNAc(OH) and AcNeu in this peak, as well as the identity of the tetra- and trisaccharides were confirmed by paper chromatography in two solvent systems; *n*-butylacetate/glacial acetic acid/water (3 : 2 : 1, v/v) and pyridine/ethyl acetate/water/glacial acetic acid (5 : 5 : 3 : 1, v/v) [1].

The nature of the carbohydrate moieties of this glycoprotein (IIB3b′W) was further explored by treating the asialoglycoprotein (prepared by mild acid hydrolysis) with endo-α-*N*-acetylgalactosaminidase. The released product, consisting of 87% of the radioactivity in the asialocompound, co-eluted on a Bio-Gel P-2 column with Gal(1 → 3)GalNAc prepared by treatment of asialofetuin glycopeptide with the same enzyme [19]. The radioactive product was isolated, desalted by passage through mixed bed ion exchange and examined by paper chromatography

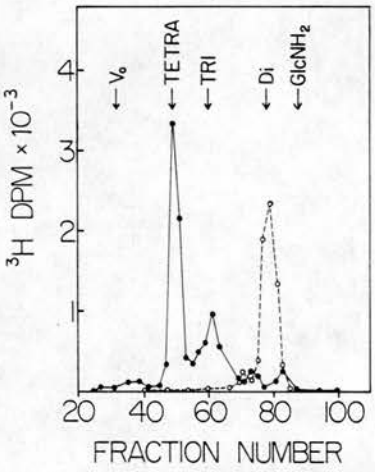



Fig. 4. Gel filtration of IIB3b′W after alkaline borohydride treatment. The glycoprotein IIB3b′W was treated with alkaline borohydride (●——●) and chromatographed on a BioGel P-6 column (0.9 × 90 cm). The column was eluted with 0.1 M pyridine/0.1 M acetic acid, pH 5.0, 1-ml fractions collected and aliquots analyzed for radioactivity. The materials eluting in fractions 47–53 and 57–67 were recovered by lyophilization and portions examined by paper chromatography. Separate portions were treated with *Vibrio cholerae* neuraminidase and re-chromatographed on the same column. In both cases, the radioactivity eluting in single peaks (○- - - - -○) was recovered by lyophilization. The peak elution positions of blue dextran ($V_0$) and reference saccharides are indicated by arrows.

using *n*-butylacetate/glacial acetic acid/water (3 : 2 : 1, v/v) as solvent [20]. The results confirmed the identity of the product as Gal(1 → 3)[$^3$H]GalNAc.

*Isolation and characterization of glycopeptides from IIB3b′W*

The glycoprotein was treated with pronase, centrifuged at 1 000 × *g* for 10 min and the glycopeptides in the supernatant examined by gel filtration on Sephadex G-50 and Sepharose CL-6B columns. On the Sephadex G-50 column, about 90% of the labeled material eluted at the void volume, indicating the presence of mostly peak A glycopeptide mentioned previously. On a Sepharose CL-6B column the material was included and eluted in the same area as the peak A glycopeptides derived from the crude glycoprotein (Fig. 3). The glycopeptide purified by chromatography on Sepharose CL-6B had no detectable [$^3$H]GlcNH$_2$; the tritium label was entirely in AcNeu and GalNH$_2$. Treatment of the glycopeptide with alkaline borohydride gave a mixture of AcNeu → Gal → (AcNeu) → GalNAc(OH) and AcNeu → Gal → GalNAc(OH). The glycopeptide was estimated to have an apparent molecular weight in the range of 12 000–15 000 by gel filtration and showed sialic acid-dependent interaction with WGA-Sepharose [7]. Redigestion of the glycopeptide with pronase did not alter its chromatographic elution profiles or its interaction with WGA.

*[$^3$H]Mannose labeling of the HM7 glycoproteins*

Plasma membranes were isolated from cells grown in the presence of [$^3$H]mannose and [$^{14}$C]glucosamine (5 μCi and 1 μCi per ml culture medium). The labeled glycoconjugates were extracted and fractionated by repeated chromatography on Sepharose CL-6B and DEAE-Sepharose columns as described above. The most acidic glycoprotein IIB3b′W, had negligible incorporation of label from [$^3$H]mannose confirming the absence of *N*-glycosidically-linked oligosaccharides. The two other glycoproteins purified, IIB2a′U and IIB2b′W, contained significant quantities of tritium radioactivity indicating the presence of *N*-glycosidically-linked oligosaccharides.

*Examination of FM13 human fetal uveal melanocytes for mucin-type glycoproteins*

The glycoconjugates were isolated by extraction of

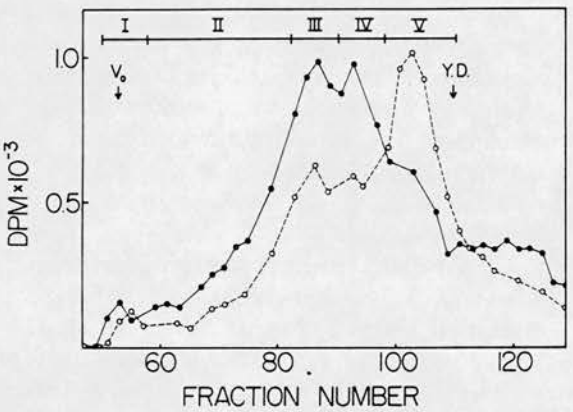



Fig. 5. Chromatography of glycoconjugates from human melanoma cells and human fetal melanocytes. Glycoconjugates isolated from HM7 human melanoma cells (●——●) and from FM13 human fetal melanocytes (○- - - - -○), metabolically labelled with [$^3$H]glucosamine, were chromatographed on Sepharose CL-6B column (1.5 X 100 cm) as described in Fig. 1. The results are presented by superimposing the elution profiles. The materials eluting in the area indicated by 'II' were recovered and further examined.

whole cells grown in the presence of [$^3$H]glucosamine with 0.6 M lithium diiodosalicylate/0.5% Triton X-100. The extracted glycoconjugates were partially purified by partition in an aqueous three-phase polymer system consisting of polyethylene glycol, dextran and Ficoll [21]. This step removes nucleic acids and separates (glyco)proteins according to hydrophobicity. The $^3$H-labeled glycoconjugates in the upper phase were recovered after dialysis. The elution profile of these glycoconjugates and of the glycoconjugates similarly prepared from labeled HM7 melanoma cells on a Sepharose CL-6B column is illustrated in Fig. 5. It is clear that the melanoma cells produce an increased proportion of glycoproteins eluting in the higher molecular size region (fractions 61–95) of the column, whereas the bulk of the glycoprotein from the melanocytes eluted in the lower molecular size region of the same column. These results are consistent with our earlier observation that the melanoma cultures produced a higher proportion of the larger glycopeptides than the control cells [1].

The labeled material eluting in the fractions marked 'II' was recovered and chromatographed on a WGA-Sepharose 4B column. In the case of the HM7 Fraction II, 70% of the labeled material bound to the column and was recovered by elution with 0.1 M GlcNAc. In contrast, only 49% of the labeled material of the FM13 Fraction II was bound to and eluted from the WGA-Sepharose. The WGA-interacting glycoproteins were recovered by dialysis and chromatographed on a DEAE-Sepharose column (Fig. 6). The elution of the purified $^{14}$C-labeled glycoprotein (IIB3b'W) isolated from plasma membranes of HM7 cells on the same column is also illustrated in Fig. 6.

A portion of the FM13 Fraction II (glycoproteins) was digested with pronase and the glycopeptides examined on a Sephadex G-50 column. Only 8.2% of the labeled material eluted at the void volume (peak A glycopeptide), the balance was included. This is in contrast to the elution of about 28.6% in peak A when HM7 Fraction II material was subjected to the same treatment.

## Discussion

Our major aim in these studies was the isolation and characterization of glycoprotein(s) rich in carbohydrate groups linked via *O*-glycosidic linkage to proteins. Accordingly, the five fractions obtained by chromatography on Sepharose CL-6B (Fig. 1)

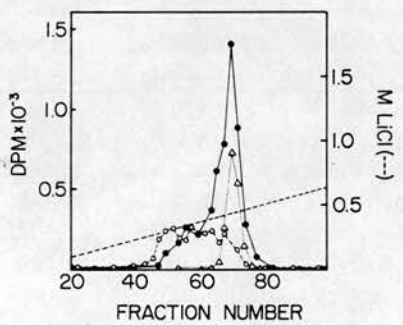



Fig. 6. Chromatography of melanoma and melanocyte glycoproteins (fraction II) on DEAE-Sepharose. The fraction II glycoproteins from melanoma cells and melanocytes (Fig. 5) were purified by affinity chromatography on WGA-Sepharose. The WGA-interacting glycoproteins from melanoma (●——●) and melanocyte (○- - - - -○) were each mixed with $^{14}$C-labeled glycoprotein IIB3b'W (△· · · · · △) and chromatographed on DEAE-Sepharose column (1.5 X 18 cm). The column was eluted with a linear gradient of 0.1 to 1.0 M LiCl in 50 mM Tris-HCl, pH 8.0, containing 0.1% Triton X-100 and 0.1 mM toluenesulfonyl fluoride. Fractions of 1 ml were collected and analyzed for radioactivity. The results are presented by superimposing the elution patterns.

were examined for the presence of WGA-binding glycopeptides (GPIa and GPIb) previously described [1].

Purification of fraction II using buffers containing 0.1% Triton X-100 and 0.1 mM toluenesulfonyl fluoride yielded three apparently homogenous glycoproteins. The recovery of the glycoproteins was poor in the absence of detergents, apparently due to aggregation and precipitation [22]. The molecular weight of the most acidic glycoprotein (IIB3b′W) was estimated as 100 000 by gel filtration [22]. The gel electrophoresis data indicate a smaller subunit molecular weight, but the anomalous behavior of glycoproteins in the presence of dodecyl sulfate [23] does not permit an accurate estimation by this technique.

The sialic acid and hexosamine analysis indicated that the glycoprotein which had the highest affinity for DEAE-Sepharose (IIB3b′W) had the highest sialic acid and GalNAc contents, whereas the glycoprotein (IIb2a′U) with the least affinity had the lowest sialic acid and highest GlcNAc content. The low sialic acid content of this glycoprotein is also consistent with its noninteraction with WGA-Sepharose [7]. The isolated glycoproteins may be considered as representative of three general classes of glycoproteins, containing only *N*-glycosidically linked or only *O*-glycosidically linked or both types of oligosaccharides [24]. These macromolecules did not contain detectable $^{35}S$ radioactivity and therefore, are not hybrid glycoprotein-glycosaminoglycan molecules of the type described by Baker et al. [25].

The high galactosamine content of IIB3b′W and the lack of mannose suggested that this glycoprotein had a mucin-type carbohydrate structure. The *O*-glycosidically-linked oligosaccharide was shown to be mainly tetra- and trisaccharides of the type $(AcNeu)_{1\ or\ 2} \rightarrow [Gal \rightarrow GalNac(OH)]$.

To the best of our knowledge, an intrinsic membrane glycoprotein having carbohydrate entirely *O*-glycosidically linked to the protein has not been previously described. Several membrane glycoproteins rich in mucin-type oligosaccharides but also having serum-type oligosaccharides are known. These include glycophorin ($M_r$ 31 000) from human erythrocyte ghost [26,27], a glycoprotein ($M_r$ about 100 000) from plasma membranes of ascites hepatoma AH 66 [12,28], bovine [29] and porcine [30] erythrocyte membrane and thyroid plasma membrane [31] glycoproteins. An extrinsic mucin-type glycoprotein (epiglycanin) has been isolated from TA3 Ha mouse mammary carcinoma cells and extensively investigated by Codington and co-workers [32,33]. In preliminary experiments, the human melanoma glycoprotein IIB3b′W failed to complex with rabbit anti-human glycophorin antibodies (Bhavanandan, V.P., unpublished data).

The FM13 melanocytes produced significantly lesser proportions of the WGA-binding glycoprotein identical or similar to IIB3b′W (Fig. 6). This is in agreement with our previous results that pronase digests of cultured human fetal uveal melanocytes or spent culture medium yielded markedly reduced quantities of the WGA-binding mucin-type glycopeptides GPIa and GPIb [1].

In future investigations we hope to isolate more quantities of the described glycoproteins by extraction of membranes prepared from HM7 tumors grown in athymic mice for additional biochemical and immunological characterizations.

## Acknowledgements

We are most grateful to Drs. B.C. Giovanella and K. McCormick (St. Joseph Hospital, Houston, TX) for providing the human fetal melanocyte culture. This work was supported in part by United States Public Health Service Grants CA 15483 and CA 17686.

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# CHAPTER II

# CELL SURFACE GLYCOPROTEIN MARKERS FOR NEOPLASIA

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## I. Introduction

It is generally recognized that events which occur at the surface of eukaryotic cells can regulate a variety of phenotypic characteristics. These include essential functions such as transport of key metabolites and re-




ISBN 0-12-147679-0

sponse to external stimuli which may allow a cell to move from a quiescent or resting state through the cell cycle to mitosis. These events are modulated by a wide variety of receptor molecules which are either integrally or transiently associated with the plasma membrane and provide specific recognition sites for appropriate ligand interactions. It is not surprising, therefore, that cells which have undergone malignant transformation or otherwise lost aspects of growth control have associated with their external membrane, either continually or intermittently, molecules unique to their particular growth characteristics; (for reviews see Wallach, 1968; Burger, 1973; Emmelot, 1973; Hughes, 1973, 1976; Warren *et al.*, 1974; Glick and Santer, 1978; Yamada and Pouyssegur, 1978; Hynes, 1979). In some situations these serve as do other cell surface molecules, in a receptor capacity to respond to external signals in such a fashion as to promote cell division or regulate other metabolic events. The binding of molecules (such as insulin, steroid hormones, epinephrine, etc.) can induce either conformational changes within the membrane leading to altered activities or to internalization of the receptor ligand complex with eventual targeting for the nucleus of the cell thus regulating expression of particular genes. The immense diversity of signals to which a cell must respond requires the presence on its surface, therefore, of a broad variety of receptor type molecules. Most, if not all, of these are glycoproteins for reasons which are physiologically and biologically appealing. There are a diversity of carbohydrate molecules available. The hydrophilic nature of the sugars provides the necessary directionality for these molecules ensuring that the appropriately substituted regions of the polypeptide are directed toward the outer environment. In addition, there is sufficient opportunity for structural variation to provide the level of selectivity and specificity necessary to discriminate among a broad variety of external signals. Normally there are only a few sugars found in mammalian glycoproteins but the opportunities for branching and for differing distributions on any given polypeptide backbone provide an enormous number of possible structures. For a trisaccharide containing three different sugars, for example, there are well over a thousand possible structures whereas for a comparable tripeptide only six structures exist.

## II. Glycoprotein Structures

In the sense that a genetic control is exerted over the structures of surface macromolecules, the glycosyl or saccharide portion of these is constructed by addition of mono- or oligosaccharides to a growing polypeptide chain at specific attachment sites. As a rule, only two types of

carbohydrate–peptide linkage are found in glycoproteins associated with the plasma membrane (Marshall and Neuberger, 1970; Spiro, 1970; Marshall, 1974).

The first is linkage between the amide nitrogen of asparagine and a carbohydrate chain wherein the initial sugar attached to the polypeptide is *N*-acetylglucosamine. Such oligosaccharide attachments are formed by a complex biosynthetic pathway involving the addition of a 14 unit oligosaccharide (dolichol-P-P-*N*-acetylglucosamine$_2$ mannose$_9$ glucose$_3$) *en bloc* to specific asparagine residues within the polypeptide chain (Struck and Lennarz, 1980). Partial sequence studies have indicated that asparagine residues are candidates for glycosylation when they are separated from serine or threonine residues by one amino acid whose structure appears not to be regulated (Asn-X-Ser). Other asparagine residues are not glycosylated, and even those meeting sequence requirements may not be glycosylated; regulation in this area is still a matter of continuing study. Processing of the 14 member oligosaccharide unit takes place first by removal of the glucosyl moieties followed by trimming of the mannose structure and subsequent elongation to provide one of two types of final structures: either an oligomannosyl (so-called simple) type or the lactosaminyl (complex) types which generally terminate in a sequence of *N*-acetylglucosamine-galactose-sialic acid (see Fig. 1). The branching possibilities allow for the latter group to have as many as four such end terminal units attached to a single glycosyl core. Thus, the oligosaccharide structures may

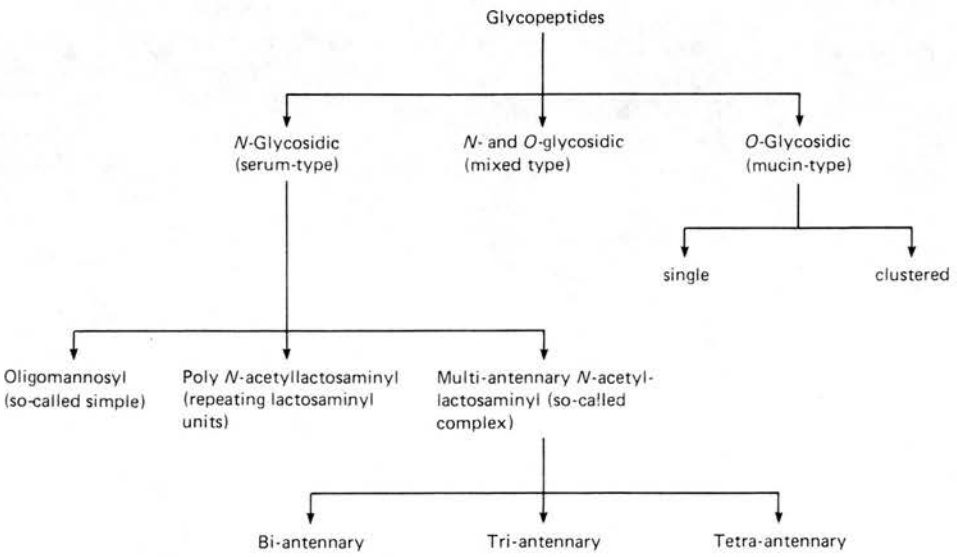


FIG. 1. Types of cell surface glycopeptides presently known.

contain up to about 20 monosaccharide units, with single or multiple antennary structures giving a wide diversity of possible products. As a general rule, sialic acid (and fucose) residues are terminal nonreducing ends and provide a stop signal for further glycosylation. The diversity of glycosyltransferases required for the elongation of processed oligosaccharide chains is considerable and few of them have been studied in detail. Regulation is not thoroughly understood and it is believed, at least by some investigators, that the control is imperfectly established and that considerable microheterogeneity exists in the end product carbohydrate chains, especially with regard to completeness of glycosylation at the outer periphery.

A second type of carbohydrate peptide linkage that is found in these glycoproteins is that between the hydroxyl group of either serine or threonine residues and *N*-acetylgalactosamine which serves as the initiating residue for oligosaccharide chain assembly. In this case, the glycosyl transfer appears to take place by sequential addition of monosaccharides from nucleotidyl sugars with galactose usually the next sugar and sialic acid commonly present. The structures found for this class of oligosaccharides may be rather simple as are present in erythrocyte glycophorin wherein two sialic acids, one galactose and one *N*-acetylgalactosamine, comprise the entire oligosaccharide unit (Lisowska, 1969; Thomas and Winzler, 1969), to those found in epithelial secretions of intestinal mucosal cells or lung cells wherein large complex oligosaccharide chains containing up to 15 sugars are present. It is not uncommon for the *O*-glycosidically linked carbohydrate chains to bear blood group determinants, especially if the individual is a secretor. However, these are less frequently found on cell surfaces than they are in secretory products. There is little or nothing known about the regulation of the end product structure for the *O*-glycosidically linked glycoproteins. Thus, structural diversity or heterogeneity is the norm and a single family of molecules may be derived from a common polypeptide chain with a considerable diversity of carbohydrate substitution. The recognition of appropriate serine and threonine residues by the glycosyltransferases is not understood nor is the control of oligosaccharide chain length. It is generally believed that this is a kinetic regulation involving transit time in the Golgi apparatus for the incompletely glycosylated protein and availability of the nucleotidyl substrates to the appropriate membrane-bound glycosyltransferases. Thus, at a given serine or threonine residue in such a glycoprotein, glycosyl substitution may or may not have taken place and the chain may be completed to different sizes.

Accordingly, isolation techniques must compromise in terms of purity criteria, generally accepting overall compositional analyses as representa-

tive rather than applying the standards usually employed for simple proteins. Single molecule purity probably does not exist in this class of compounds and structural analyses represent probability statements rather than specific details. The methodologic problems involved are discussed later in this chapter but are formidable in terms of defining both the oligosaccharide and polypeptide structures in detail (see Section VII).

## III. Qualitative and Quantitative Changes

Association with the transformed state can be expressed in several ways. Descriptive changes involving, for example, the interaction of lectins with cell surfaces provide qualitative or crude information about some alteration in surface properties (Nicolson, 1974). Whether this represents the expression of new molecules, modification of old ones, total or local bilayer fluidity change, or some cryptic phenomenon whereby previously available macromolecules are now physically reorganized within the membrane structure such that the external probes no longer can find them has generally not been defined. The same type of problem applies to surface alterations detected by electron microscopy using, for example, ferritin-conjugated lectins or antibodies. There is an element of qualitative information that is definitely available from such studies but it is impossible to translate such observations to the presence or absence of specific molecular species. Similarly, changes in biologic properties have been correlated with the presence or absence of specific types of macromolecules on the cell surface. A well-studied example is the mouse mammary carcinoma TA3 system wherein transplantability across species boundaries is associated with the presence on the cell surface of an unusual glycoprotein (epiglycanin) whose apparent function is to mask otherwise responsive cell surface antigens (Codington, 1975; Codington *et al.*, 1978, 1979a,b; Cooper *et al.*, 1979). Properties such as adhesion to substrate, metastatic potential, and immunogenicity of whole cells as well as masking have been correlated with the presence on the cell surface of particular types of macromolecules (Hudson and Kunz, 1925; Hynes, 1976; Codington, 1978). Specific molecules have not been identified and structural information not forthcoming to define their nature.

A type of quantitative change that has been widely associated with malignancy is the loss of a large glycoprotein from the cell surface. This macromolecule, originally described about 10 years ago (Hynes, 1973, 1976), has been the subject of intensive study. A variety of names have been used including fibronectin, LETS (large, external, trypsin or transformation sensitive), cell surface protein, and galactoprotein A among others (Yamada and Weston, 1974; Vaheri and Ruoslahti, 1975; Hako-

mori, 1975; Engvall and Ruoslahti, 1977; Hynes *et al.*, 1979). The near identity of this material with a previously described circulating glycoprotein, cold insoluble globulin, has also been well documented and the relationship between the surface glycoprotein and the circulating form has been examined in some detail (Mosesson and Umfleet, 1970; Ruoslahti and Vaheri, 1974; Mosesson *et al.*, 1975; Mosher, 1975). The generality of the loss of fibronectin from transformed cell surfaces is broad but not total and may well be related to the generally increased protease activity associated with malignant tumors (Unkeless *et al.*, 1973; Rifkin *et al.*, 1974; Lang *et al.*, 1975). This field has been extensively reviewed in recent years and insofar as fibronectin represents a cell surface glycoprotein marker for neoplasia, it can only be said to represent a negative marker since it is a normal component of all fibroblastic cells and a number of other cell types that have been studied.

The specific associations of glycoproteins with discrete types of malignant cells have been under intensive study for well over a decade. The literature is frequently and extensively reviewed and the following discussion does not claim to be exhaustive but rather representative. The topical subdivision is arbitrary and other means of classification could well have been employed. We will discuss initially studies with virus-transformed cells, second, animal cells and animal tumor models, data available from studies with human systems, and finally the methodology.

## IV. Virus-Transformed Cells

The association of viruses with animal tumors has been known since the description of Rous sarcoma virus nearly 70 years ago (Rous, 1911). That virus-directed transformation would be an excellent model to study virtually every event associated with cellular metabolism is also well recognized and a wide variety of systems has been studied. Reviews have been published recently by Montagnier and Torpier (1976) and on RNA tumor viruses by Kurth (Kurth and Bauer, 1975; Kurth, 1976; Kurth *et al.*, 1979).

Early studies with Rous sarcoma virus transformed cells indicated that glycopeptides derived from such cells had increased molecular size primarily due to either increased fucosylation or increased sialylation and that there was an increase in sialyltransferase levels associated with the transformed cell (Warren *et al.*, 1972a,b). At least one glycosyltransferase was demonstrated to be externally directed as contrasted with the same activity in nontransformed cells (Bosmann 1972; Bosmann *et al.*, 1974) suggesting that accessibility of the external glycoproteins to this enzyme

could result in altered glycosylation patterns. The problem of substrate availability in terms of nucleotidyl sugars has rarely been addressed in any of these studies. The loss of LETS glycoprotein from Rous sarcoma virus-transformed cells has also been studied in some detail. It is generally agreed that the levels of this glycoprotein are sharply reduced when chick embryo fibroblasts are transformed with Rous sarcoma virus (Hynes and Wyke, 1975; Parry and Hawkes, 1978). In an attempt to define tumor-specified cell surface changes more closely, transformed fibroblasts have been examined by both metabolic labeling and labeling of cell surface components by lactoperoxidase catalyzed iodination (see Section VII).

Early experiments carried out by Warren with control and Rous sarcoma virus transformed BHK 21 cells indicated that although the glycopeptides present in the latter cells appeared to be of somewhat larger size, treatment with sialidase converted these to a size pattern characteristic of the controls (Warren *et al.*, 1972a). Accordingly, it was concluded that the major difference found in the transformed cell population could be ascribed to the increased sialylation of normally present glycoproteins; these results were later shown to be due to an increase in multiantennary saccharide substitution. A number of related experiments have subsequently been carried out along these lines including examinations of fucosylated glycopeptides with comparisons made between the pattern of glycopeptides obtained from metabolically labeled material (tritiated sugar material) and molecules accessible on the cell surface by one of several labeling procedures such as lactoperoxidase catalyzed iodination, periodate oxidation–sodium borotritide reduction, or galactose oxidase–sodium borotritide reduction with or without prior neuraminidase treatment (Critchley *et al.*, 1976, Gahmberg *et al.*, 1976). The primary observations can be summarized as follows: in general, the glycopeptides of the transformed cells appear to be larger. The size differences are attributable in part to increased sialylation but the fucose-containing glycopeptides appear to be somewhat larger as well even after neuraminidase treatment (Glick, 1979). Both *N*- and *O*-linked glycopeptides appear to be present with the latter group clearly less prevalent in the untransformed cells (Santer and Glick, 1979; Hunt *et al.*, 1981). Examination of radiolabeled material either from metabolic labeling experiments or by cell surface labeling indicates substantially identical patterns although Flowers has reported some change in molecular size and an increase in a 150,000 molecular weight glycoprotein (Flowers and Glick, 1980), and new surface antigens have been described (Phillips and Perdue, 1978). Increased sialic acid is generally present after transformation but explicit isolation of altered or modified glycoproteins has rarely been carried out. It is believed

that the virus-transformed cells show an increased turnover of cell surface glycoproteins, including not only fibronectin but others more tightly associated with the plasmalemma. Rieber has indicated that although the general turnover of cell surface proteins in transformed cells is increased, there are variations within this group and that the changed rates are not all uniform (Rieber *et al.*, 1975).

Similar studies have been carried out with a wide variety of virally transformed cells including polyoma (Chiarugi and Urbano, 1972; Gahmberg *et al.*, 1974; Ogata *et al.*, 1976; Koide *et al.*, 1979), SV40 (Wu *et al.*, 1969; Meezan *et al.*, 1969; Onodera and Sheinin, 1970; Culp and Black, 1972; Sheinin and Onodera, 1972; Ceccarini, 1975; Smets *et al.*, 1976; Luborsky *et al.*, 1976; Onodera *et al.*, 1976; Duksin and Bornstein, 1977; Aoi and Yokota, 1980), and murine sarcoma viruses (Fishman *et al.*, 1976; Van Nest and Grimes, 1977; Kamm and Grimes, 1978), mouse mammary tumor viruses (Yang *et al.*, 1977; Teramoto *et al.*, 1977), leukemia viruses (Smart and Hogg, 1976; Ledbetter and Nowinski, 1977; Troy *et al.*, 1977; Murray and Kabat, 1979), and Epstein–Barr virus (Strnad *et al.*, 1981). In virtually every case, some alteration in surface labeling patterns has been observed including the appearance of viral envelope glycoproteins. Many of the recent studies depend on surface accessibility of glycoproteins using either the periodate–borotritide or galactose oxidase techniques. It should be noted that these procedures, especially the latter, will label only available surface glycoproteins and that those components which are masked or rapidly shed may appear to have been altered when in fact their physical configuration in association with the plasma membrane may be that which is changed.

Several studies have indicated the obverse of these general conclusions noted above. It was reported that polyoma virus-transformed BHK cells have associated with the plasma membrane, glycoproteins which tend to be less glycosylated than those found in the control population whereas those released into the medium appear to be more heavily glycosylated (Chiarugi and Urbano, 1972). Conversely, using the same system and cells metabolically labeled with mannose, the mannose-containing oligosaccharides were isolated after pronase digestion, separation of glycopeptides, and cleavage of the oligosaccharides with endoglycosidase H. It was concluded that the oligomannosyl fragments were substantially the same in the normal and transformed cells (Koide *et al.*, 1979).

In a study with polyoma-transformed cells, the differences in the transformed glycopeptides were shown to involve more than simply sialic acid as observed by affinity chromatography on Con A–Sepharose (Koide *et al.*, 1979). The different patterns of affinity chromatographic behavior in-

dicated that although the core structures of the glycopeptides were similar, the outer branches of the transformed cells were more complex. This result is in keeping with those generally obtained with Rous sarcoma virus-transformed cells which indicate that the glycosylation pattern tends to become more complete or extensive yielding glycopeptides which are both fully sialylated and of a higher molecular size.

A number of investigators have examined SV40-transformed cells utilizing basic strategies very similar to those employed in other systems. Two types of studies indicate differences between control (3T3) cells and their SV40 transformed counterparts: the first involves affinity chromatography utilizing as ligand antibodies elicited by whole cells or plasma membranes. As a rule, cells are either metabolically or surface labeled and glycoproteins that adsorb to the affinity matrix eluted by appropriate measures and examined by polyacrylamide gel electrophoresis. Glycoproteins have been identified with molecular weights in the 50,000–60,000 range; these appear to be associated with SV40 transcription, although detailed characterization has not been carried out (Luborsky *et al.*, 1976). One report has indicated that exposure of SV40-transformed cells to tunicamycin affects surface glycosylation patterns resulting in a marked reduction of glycoprotein affinity for Con A–Sepharose. This is not surprising since tunicamycin inhibits an early step in the assembly of the dolichol oligosaccharide which serves as the precursor for all of the *N*-glycosidically linked oligosaccharides found on glycoproteins. A second approach involves genetic analysis. The geneA of SV40 was shown to be related to a sialyltransferase and thus to associated changes in glycosylation (Onodera *et al.*, 1976). The implication from these data is that the expression of a new type of sialyltransferase in the transformed cells allows modification of preexisting glycoproteins to take place with associated increased sialylation—a frequently observed result and one consonant with alterations in both chromatographic mobility and lectin affinity behavior.

Work with mouse mammary tumor virus-transformed cells has indicated the presence of a 52,000 molecular weight externally directed glycoprotein which appears to be uniquely associated with the transformed cell (Yang *et al.*, 1977; Teramoto *et al.*, 1977). This glycoprotein has been identified both by immunologic means and also by direct visualization following lactoperoxidase catalyzed iodination and gel chromatography. It is not known if this protein arises as a result of expression of a viral gene or due to a secondary circumstance wherein a normally quiescent host gene is expressed following viral transformation. Tumor-specific antigens have also been described for spontaneous or chemically transformed cells (Invernizzi *et al.*, 1977; Natori *et al.*, 1977, 1978).

## V. Animal Tumors and Derived Cell Clones

### A. Neuroblastoma

A number of laboratories have studied the C 1300 mouse neuroblastoma in cell culture (Brown, 1971; Glick *et al.*, 1976b; Glick, 1976). Differences in glycopeptide patterns between more and less differentiated cells have been indicated using metabolic labeling with tritiated glucosamine followed by trypsinization of membranes or whole cells, and chromatography on diethylaminoethyl cellulose. A study with cloned C 1300 cells involved prior induction of neurite expression with dimethyl sulfoxide or hexamethylene bis-acetamide. The cells were labeled with tritiated fucose or glucosamine and glycoproteins examined following neurite isolation from plasma membranes by shearing and discontinuous sucrose gradient ultracentrifugation. At least one qualitative difference was observed in a 200,000 molecular weight protein which appeared to be reduced in undifferentiated cell membranes but then appeared in the growth medium. This was distinct from fibronectin on the basis of molecular weight, trypsin sensitivity, and lack of growth relatedness but was a glycoprotein since it was well labeled by carbohydrate and poorly stained with Coomassie Blue (Littauer *et al.*, 1980). Further characterization of this component has not been reported.

In analogy to other studies, it has been shown that the turnover kinetics of plasma membrane components in the neuroblastoma system differ considerably for individual components (Mathews *et al.*, 1976). These studies involve carbohydrate labels such as fucose followed by gel chromatographic examination of products. As a rule, high turnover components appear to be more sensitive to protease cleavage from the plasma membrane. An increased complexity of the neuroblastoma glycopeptides as compared to those of less differentiated cells has been suggested, and in addition Glick (1976) has indicated a structural homology between both mouse and human membrane glycoproteins derived from this type of cells.

### B. Melanoma

A large number of laboratories have studied the B16 mouse melanoma system as a model for cell surface expression (Warren *et al.*, 1975; Bhavanandan *et al.*, 1977; Evans *et al.*, 1977; Yogeeswaran *et al.*, 1978; Bystryn, 1978; Gersten and Marchalonis, 1979). Early work indicated the same general pattern for these cells as observed with others in that an in-

creased content of sialic acid and fucose as well as higher molecular weight oligosaccharides was found when compared to normal tissues. It should be noted that finding appropriate controls for melanoma cells is difficult since primary cultures of readily available melanocytes (e.g., from iris), while possessing the characteristic of melanin production are not identical to the skin melanocytes; established lines of normal melanocytes are generally not available. A study of the glycopeptides produced by B16 mouse melanoma cells has indicated that an unusual sialoglycopeptide is present in these cells which has a molecular weight of approximately 10,000–12,000 and an affinity for wheat germ agglutinin–Sepharose (Bhavanandan *et al.*, 1977). The saccharide portion of this glycopeptide is analogous or identical to that found in human glycophorin and contains galactose, *N*-acetylgalactosamine, and sialic acid. The parent glycoprotein which contains this glycopeptide was also identified both from cultured cells and from the solid B16 tumor grown *in vivo* (Fareed *et al.*, 1978). An interesting feature of this glycopeptide is that its affinity for the wheat germ agglutinin–Sepharose column is completely dependent on the presence of sialic acid since digestion with neuraminidase results in cleavage of the sialic acid with concomitant loss of affinity. This is an example of lectin lack of specificity in that the glycopeptide is free of *N*-acetylglucosamine which is not only the nominal ligand for wheat germ agglutinin but also can be used as a haptenic displacer to remove the bound glycopeptide from a WGA–Sepharose column (Bhavanandan and Katlic, 1979).

The availability of sublines of the B16 with high and low metastatic potential has prompted a number of studies designed to examine the relationship between cell surface components and metastatic behavior. In general, the more metastatic lines have been found to have increased concentrations of sialoglycopeptides and possibly some additional glycoproteins which are not present in low metastatic lines (Warren *et al.*, 1975; Nicolson *et al.*, 1977; Raz *et al.*, 1980b). Electron microscopic visualization of high and low metastatic lines of B16 has been carried out using cationic ferritin derivatives. Qualitative differences were observed although it was reported in these studies that the same level of sialic acid was present in both lines (Raz *et al.*, 1980a). The difference between total sialic acid content and the rather descriptive nature of the electron microscopic visualization makes it difficult to interpret these data further. As a rule, the glycoproteins in high and low metastatic lines were similar but at least one (78,000 molecular weight) sialoglycoprotein was reported to be related to the ability of these cells to colonize in the lung (Jumblatt *et al.*, 1980). Glycoproteins from these same cells showed a reduced affinity for wheat germ agglutinin–Sepharose. There appeared to be no differences in

membrane fluidity, substrate adhesion properties, or ganglioside patterns. A directly contrasting report has indicated that increased levels of sialic acid are well correlated with metastatic potential for these cells and that this increased level of sialic acid is related to increased lectin affinity. In a continuation of this type of study, an examination was made of B16 cells which are resistant to wheat germ agglutinin. Variants were selected by growth in high concentrations of the lectin and the surface glycopeptide patterns examined by conventional techniques. There is a reduction in sialic acid containing glycopeptides both of the *N*- and *O*-glycosidic type with an apparent increase in fucose-containing glycopeptides (Finne *et al.*, 1980b). It was concluded that the glycopeptide patterns indicate that more than one receptor for wheat germ agglutinin is present on the surface of these cells and that in different variants one or another such receptor might be present in reduced quantity. In addition, it was suggested that a certain minimal number of wheat germ agglutinin molecules must bind to the surface of such cells before a toxic threshold is crossed. Thus, reduction in a single type of glycoprotein receptor might be sufficient to render cells resistant to the lectin (Lin and Davidson, 1981). There was some suggestion that the resistant cells had less metastatic and tumor-forming potential than did the parent lines but the *in vivo* studies were insufficient to allow general conclusions to be drawn. It should be noted that studies in other laboratories have indicated, at least intermittently, that alteration of cell surface sialic acid content by pretreatment with neuraminidase may render such cells either more immunogenic or less tumorigenic depending on the nature of the host response (Currie *et al.*, 1968; Simmons and Rios, 1971).

### C. Ascites

Detailed studies have been done on the general profile of plasma membrane glycoproteins obtained from cells grown in ascites form including Ehrlich ascites carcinoma (Nachbar *et al.*, 1976), sarcoma 180 (Shin and Carraway, 1973), and mouse mammary carcinoma (Codington *et al.*, 1979a,b; Sherblom and Carraway, 1980). As a rule, plasma membranes have been isolated utilizing the zinc chloride procedure developed by Warren (Warren *et al.*, 1966; Colombini and Johnstone, 1973) and proteins solubilized from such preparations with detergents such as deoxycholate, lithium diiodosalicylate, or by sequential extraction with high salt followed by detergent. The solubilized materials are then subjected to affinity chromatography on lectin Sepharose columns utilizing lectins such as *Ricinus communis*, wheat germ agglutinin, concanavalin A, soybean

lectin, and limulin. Glycoproteins have been identified from Ehrlich ascites cells ranging in molecular weight from 35,000 to 200,000 (Nachbar *et al.*, 1976). However, there was no comparison of such material with any control cell so it is impossible to tell whether any qualitative or even quantitative changes occurred. A second study with sarcoma 180 cells utilized lactoperoxidase-catalyzed iodination of plasma membranes followed by their isolation and solubilization. Glycoproteins were identified which ranged in molecular weight from 33,000 to 300,000 (Shin and Carraway, 1973). Utilizing the AH 66 hepatoma, proteins were isolated from the plasma membrane of these by lithium diiodosalicylate extraction followed by phenol partitioning. A protein was identified with a molecular weight of 165,000 which had approximately 50% carbohydrate, most if not all of which was *O*-glycosidically linked (Funakoshi and Yamashina, 1976; Nakajo *et al.*, 1979); it was indicated that the glycoprotein was glycophorin-like. It should be noted that glycophorin-like macromolecules have now been reported to be surface components of a number of cells and at least in the case of the B16 mouse melanoma, the saccharide structures have been sufficiently well identified to confirm this similarity (Bhavanandan *et al.*, 1977). The immunologic reactivity of cell surface glycoproteins with antiglycophorin antibodies has been examined and our group has indicated the presence of reactive surface molecules using indirect immunofluorescent techniques (Barsoum and Bhavanandan, 1981, unpublished results).

### D. Hepatoma

A number of hepatoma lines have been examined for glycoprotein alterations; most studies relied on general descriptions. In Reuber and Novikoff hepatoma cells, neuraminidase-sensitive sialic acid is increased in these cells as compared to control liver cells, as is the overall level of sialic acid and glycopeptide size (Glenney and Walborg, 1979). These data are generally consistent with reports from other cell studies. An early report indicated that treatment of AH 62 hepatoma cells with either concanavalin A or neuraminidase affected the electrophoretic mobility of these cells (Yamada and Yamada, 1973). This simply indicates that charged moieties responsible for electrical migration are either available to the lectin and thus masked by its presence or susceptible to enzymatic cleavage, since removal of sialic acid from cell surface macromolecules would be expected to influence mobility.

In studies with Novikoff hepatoma cells, descriptive profiles have been developed following lactoperoxidase-catalyzed iodination and lectin affin-

ity chromatography. More detailed studies were performed following labeling of sialic acid residues by periodate treatment and borotritide reduction followed by examination of detergent solubilized glycoproteins on SDS–polyacrylamide gels. A variety of proteins ranging in molecular weight from 25,000 to 240,000 was detected with representatives at 120,000 and 92,000 which contain both sialic acid and *N*-acetylgalactosamine as indicated by affinity of those macromolecules for soybean lectin following neuraminidase treatment (Glenney *et al.*, 1979).

Morris hepatoma cells in contrast to normal liver have increased plasma membrane fucose (4-fold) as well as an increase in the number of fucose-labeled glycoproteins (Vischer and Reuter, 1978). Specific macromolecules, however, were not identified in these studies. In still other hepatoma work, trypsin-sensitive membrane glycoprotein fragments were isolated from HTC cells following either cell surface labeling with lactoperoxidase or metabolic labeling using leucine and fucose. A heavily sialylated product of molecular weight 55,000 was identified and noted to contain approximately 17 sialic acid residues per mole (Baumann and Doyle, 1979). This was apparently a fragment derived from an 85,000 molecular weight precursor containing about 40% carbohydrate. Although both of these were purified, further characterization was not carried out. The relation of these to specific surface markers of hepatoma remains to be defined. In related work, AS 30D hepatoma cell glycoproteins were examined by several labeling techniques followed by affinity chromatography on *Ricinus communis* lectin–Sepharose and two-dimensional gel electrophoresis. Three galactoproteins were identified which possess charge heterogeneity due to varying levels of sialic acid. One of these was cleaved to a 63,000 molecular weight fragment by papain but the relationship, if any, of this to the above mentioned 55,000 molecular weight component was not defined (Glenney *et al.*, 1980).

### E. Mammary Carcinoma

A detailed study of the surface properties of a mouse mammary carcinoma (TA3) system has been carried out by Codington and co-workers. Two variants of this mammary carcinoma differ in their ability to be transplanted into allogeneic hosts. It has been found that the transplantable form contains on its cell surface, a carbohydrate-rich glycoprotein called epiglycanin which is not present in the nontransplantable line. This protein has a molecular weight of approximately 500,000 and contains about 80% carbohydrate, essentially all of which is *O*-glycosidically linked. There are approximately 500 to 600 carbohydrate substitution sites on the

polypeptide chain with most of the subsituents being disaccharide units. The apparent function of this macromolecule, which is broadly distributed over the cell surface, is to mask surface antigenic structures which may permit immune response of the host and thus rejection of the tumor cells. The saccharide units are free of fucose and it has been shown that removal of sialic acid results in a decrease in transplantability of the tumor cells (Sanford, 1967). This is probably the best studied of the cell surface glycoprotein markers and its function is reasonably well established. There is some saccharide heterogeneity so that structural data represent composite information but the overall features are quite clear and have been confirmed by direct electron microscopic visualization (Slayter and Codington, 1973; Miller *et al.*, 1977).

A related study using a rat mammary carcinoma (13762) grown in ascites form has focused on membrane glycoproteins solubilized either by detergent extraction or by treatment with guanidine hydrochloride. Purification included cesium chloride density gradient centrifugation and led to the isolation of one mucin type glycoprotein which may serve a masking function as does epiglycanin in the mouse system (Sherblom *et al.*, 1980). It was noted that within the membrane system, this component and a second glycoprotein were associated to give a complex with a molecular weight of approximately 600,000 (Sherblom and Carraway, 1980). One subunit bound to concanavalin A and one did not. The nature of the association was not covalent since the two species were separable in the presence of guanidinium chloride; the overall data suggest that the association is primarily hydrophobic.

### F. Lung Carcinoma

Studies with a murine lung carcinoma revealed the presence of a surface glycoprotein with a molecular weight of 180,000 which is not present on normal cells (Kennel, 1979; Kennel *et al.*, 1980). The protein was identified by metabolic labeling, isolated, and a radioimmunoassay developed. The data suggest that the protein is shed from the surface of the cells at some regular rate and eventually appears in the serum as the tumor burden in the animal increases. Lactoperoxidase-catalyzed iodination of the cell surface indicated that the molecule was expressed at least transiently at that locus; comparable macromolecules were identified in several lung carcinoma lines. There was some suggestion that charge heterogeneity was present based on isoelectric focusing studies but apparently this was not due exclusively to variations in sialic acid (Eisinger and Kennel, 1981).

## G. Leukemias and Lymphomas

Mouse leukemia cells (GRSL) were shown to contain a glycoprotein with apparent *N*-acetylgalactosaminyl residues, a conclusion based on interaction with a lectin from *Dolichos biflorus* (Muramatsu *et al.*, 1980). This glycoprotein had a molecular weight of 100,000, contained *O*-linked oligosaccharides, and had properties somewhat reminiscent to one described by Bramwell and Harris (Bramwell and Harris, 1978a,b). In a number of other studies on leukemic cell lines, properties of glycopeptides have been described. Increased size and fucose content was generally noted and interaction with lectins defined (Hourani *et al.*, 1973).

An interesting report suggested that antigenic determinants of membrane glycoproteins derived from a mouse plasma cell line may involve *N*-acetylneuraminic acid (Prat *et al.*, 1975; Prat and Comiglio, 1976). These studies were based on immunologic methods with alterations in immune reactivity observed following treatment with neuraminidase. In spite of these observations, the interpretation is unlikely to be correct. As a rule, sialic acid does not function as an antigenic determinant although its presence in a molecule can confer specific conformations which make one or another antigenic determinant site unique and accessible. Thus, although removal of sialic acid from a given glycoprotein may alter immune properties (similar to studies carried out with MM and NN glycophorin) (Springer and Desai, 1975; Lisowska and Wasniowska, 1978; Sadler *et al.*, 1979) this does not necessarily mean that the sugar itself is recognized by the antibody but rather that its removal occasions a conformational change which results in altered immune properties.

## H. Viral Glycoproteins

A number of laboratories have been concerned with the possible expression of viral glycoproteins on the surface of infected cells (Nilsson *et al.*, 1977; Snyder and Fleissner, 1980; Kurth *et al.*, 1977; Kurth and Mikschy, 1978; Krakower and Aaronson, 1978; Krakower *et al.*, 1978; Mesa-Tejada *et al.*, 1978). Attempts have been made to define such components immunologically as well as by direct analysis of labeled macromolecules. Although the results are suggestive, perhaps the clearest data have come from studies with Epstein–Barr virus glycoproteins. Two types of glycoproteins have been identified, one on a human lymphoma line and one as a membrane antigen from infected cells (Nilsson *et al.*, 1977). The labeling techniques involved either galactose oxidase or neuraminidase and then galactose oxidase; glycoproteins of molecular weight 71,000 or 69,000 and 236,000 were identified. The latter was characterized

on the basis of lectin affinity and subsequently isolated on a preparative scale by polyacrylamide gel electrophoresis.

### I. General Phenomena

A provocative series of studies has been carried out by Bramwell and Harris on several murine lines, including hybrid lines with suppressed malignancies (Bramwell and Harris, 1978a,b). Plasma membranes were isolated from these cells by treatment with zinc chloride followed by discontinuous sucrose gradient centrifugation. The membranes were extracted with Triton and the glycoprotein patterns examined by lectin-binding properties and gel chromatographic behavior. A membrane-associated glycoprotein with a molecular weight of approximately 100,000 and an isoelectric point of approximately 4 was found with high consistency in the tumorigenic cells. The level of this component was reduced in the relatively less malignant hybrid lines. It may be present in the cell membrane as a dimer; there was some suggestion, not confirmed, that this molecule is associated with glucose transport.

## VI. Human Cells

A number of human cell lines have been examined for specific membrane-associated glycoproteins (Table I).

### A. Melanoma

Early work conducted with a line of melanoma cells adopted the following strategy: rabbits were immunized with inactivated human melanoma cells and the elicited antibodies used as a primary fractionation tool. Screening was conducted on extracts of solid tumors and on urine of patients for proteins which reacted with the rabbit antibodies. The reactive molecules were examined by gel electrophoresis and melanoma associated antigens identified in the 40,000–60,000 molecular weight range (Carrel and Theilkaes, 1973).

Evidence was developed for membrane-associated melanoma antigens by use of a delayed hypersensitivity reaction utilizing extracts of melanoma cell membranes. A number of active fractions were identified although none was purified (Hollinshead *et al.*, 1974). In comparable studies using immunologic approaches, melanoma patients were screened for the presence of circulating antibodies directed against cell surface anti-

TABLE I
GLYCOPROTEINS OF HUMAN TUMOR CELL SURFACES[a]

| Source | Methodology | Properties |
|---|---|---|
| Melanoma cells | Immunoadsorbent (patient serum) | 20,000–50,000 MW; affinity for *Lens culinaris* lectin |
| Melanoma cells | Monoclonal antibody | 97,000 MW |
| Mammary ductal carcinoma | Membrane isolation, extraction; immunoadsorbent | 20,000 MW; PCA-soluble, 38–57% carbohydrate; *pi* 4.45–5.35 |
| Lung adenocarcinoma | Standard biochemical | 77,000 MW; cross reacts with $\alpha_1$-antichymotrypsin |
| Colon adenocarcinoma | Surface labeling, lectin affinity | Carcinoembryonic antigen |
| Renal cancer cells | Metabolic labeling, two-dimensional gels | 27,500 MW; p*I* 5.7, 5.3, 4.9 |
| Neuroblastoma cells | Monoclonal antibody | 20,000 MW; fucose, glucosamine label |
| Ovarian tumor | Immunoadsorbent | 70,000 MW; PCA-soluble |

[a] See text for references.

gens of cultured melanoma cells (Carey *et al.*, 1976; Leong *et al.*, 1977a,b). In one study, a particular patient with a high titer was identified and serum from that patient utilized as a screening tool for the presence of melanoma associated cell surface antigens in a variety of cell sources (Carey *et al.*, 1979). A group of proteins in the molecular weight range of 20,000–50,000 was identified by this technique. An indication that they were glycoproteins was derived from their interaction with *Lens culinaris* lectin. However, structural details were not reported in these studies.

Surface labeling of several lines of cultured melanoma cells indicated that galactosyl residues were sterically hindered. Common features included proteins with molecular weights of 90,000 and 110,000 as well as two glycoproteins with the characteristics of Ia antigens (Lloyd *et al.*, 1979).

A putative melanoma associated antigen was identified by radioimmunoassay and a molecular weight of 97,000 was assigned (Brown *et al.*, 1981a). However, the specificity of this to melanoma was not defined, nor were any structural details reported.

Several laboratories have utilized hybridomas to develop panels of monoclonal antibodies directed against cell lines derived from human melanoma and other malignant cells (Carrel *et al.*, 1980; Dippold *et al.*,

1980). A number of common antigens have been identified but relatively few specific components. The release of antigens from melanoma cell lines has been examined but in contrast to studies with virus-transformed lines, no apparent change in antigen secretion rate was observed when data were compared to that from control cell populations (Bystryn *et al.*, 1981).

### B. Mammary Carcinoma

Studies carried out with human mammary tumors utilizing both chemical and surface localization techniques have identified tumor-related glycoproteins of approximately 20,000 molecular weight; these were distinct from carcinoembryonic antigen (Kuo *et al.*, 1973). The glycoproteins are soluble in perchloric acid and contain between 40 and 60% carbohydrate; isoelectric focusing identified a p*I* of 4.45 for one component and 5.35 for another. It was not known if these were related by differences in sialic acid content although both components contained sialic acid (Leung *et al.*, 1978; Leung and Edgington, 1980).

An association of human breast cancer with T antigen which is a precursor of the MN blood groups has been described (Springer *et al.*, 1976; Howard and Batsakis, 1980).

### C. Lung Carcinoma

An antigen associated with lung tumor has been identified as a 76,000 molecular weight, sialic acid-containing glycoprotein (Gaffar *et al.*, 1979; Braatz *et al.*, 1978). The protein was isolated by direct extraction from lung tumor and was shown to have some structural homology with $\alpha_1$-antichymotrypsin on the basis of immunologic cross reactivity; the two molecules were not identical (Gaffar *et al.*, 1980).

### D. Colon Carcinoma

The interest in carcinoembryonic antigen has prompted a number of studies with colonic carcinoma lines. Surface labeling of one such with galactose oxidase indicated the presence of at least five glycoproteins; the major one had a molecular weight of 200,000 and properties similar to those of carcinoembryonic antigen (Tsao and Kim, 1978). Soluble antigens from colonic carcinomas have been studied by the usual techniques and a number of glycoproteins identified but other than similarities to CEA, no structural details have been reported. The appearance of car-

cinoembryonic antigen on the cell surface of cultured colon adenocarcinoma cells requires protein synthesis and can be inhibited by the presence of bromouracil deoxyriboside (Rosenthal *et al.*, 1980). Internalization of the surface antigen was accomplished by exposure of the cells to antibody and the subsequently denuded cells were allowed to recover under conditions which permit reexpression of the CEA.

### E. Ovarian Carcinoma

A perchloric acid-soluble glycoprotein with a nominal molecular weight of 70,000 has been purified from extracts of human ovarian tumor (Knauf and Urbach, 1974, 1976). The development of a satisfactory radioimmunoassay has permitted discrimination between this molecule and carcinoembryonic antigen and the lack of association of this molecule with lung cancer; cross reactive material was present in colon carcinoma. The generality of this particular antigen has yet to be defined but the authors suggest that it may be common to certain types of cyst adenocarcinomas (Knauf and Urbach, 1981). Screening for the presence of this macromolecule in plasma indicates an association with advanced disease (Knauf and Urbach, 1977, 1978).

### F. Neuroblastoma

Studies on human neuroblastoma glycopeptides have shown that they have the same general features associated with those found in other transformed cells in that they tend to be more complex and perhaps more highly fucosylated (Glick *et al.*, 1976a). An unusual linkage has been identified in glycopeptides from these cells with fucose linked 1-3 or 1-4 to *N*-acetylglucosamine (Santer and Glick, 1980). At least one membrane glycoprotein with a molecular weight of approximately 20,000 has been identified from human neuroblastoma utilizing monoclonal antibodies as the detecting tool (Momoi *et al.*, 1980). Glycopeptides derived from this glycoprotein had some antigenic activity, although reactivity with glycolipids was not found. It is of interest to note that there was cross reactivity with extracts of fetal but not adult brain.

### G. Miscellaneous

Physical studies have indicated surface changes in cultured lines of bladder epithelial cells associated with transformations (Kahan *et al.*,

1977). A similar analysis compared renal cancer and normal kidney epithelial cells; protein components of molecular weights 27,500 and isoelectric points ranging from 5.7 to 4.9 were uniquely present in the malignant cells (Ogata *et al.*, 1981).

## VII. Methodology

The general approaches toward defining the nature of cell surface glycoproteins can be subdivided into metabolic or biochemical procedures as contrasted to immunologic procedures. In most cases that have thus far been described, few of the glycoproteins have been isolated in sufficient quantity to permit detailed biochemical characterization. Most studies have been performed with either metabolic or chemically labeled material and identification of unusual products by means of polyacrylamide gel electrophoresis or more recently, monoclonal antibodies. Considerable work has been done with proteolytic digests of crude fractions isolated from plasma membranes or whole cells to examine glycopeptide profiles. Progress in this field will remain limited until techniques are worked out for isolating native glycoproteins and providing at least minimal structural details for them, including immunologic behavior, so that a library of defined surface glycoproteins can be established. There are many indications that malignancies both of animal and human origin have associated with their cell surfaces glycoproteins that are either immunologically or otherwise unique; a vast amount of work remains to be done to provide adequate characterization profiles.

### A. Preparation of Cells

A major reason for the relatively slow progress in this field is that the cells utilized for studies are available in limited quantities. Developments in cell culture techniques such as growth of cells on microbeads (Levine *et al.*, 1977), in multi-plate culture systems (Schleicher, 1973), large volume fermentors and spinner cultures (Chee *et al.*, 1976), and the use of serum-free medium (Barnes and Sato, 1980a,b) should alleviate the primarily economic aspects of this shortcoming. An associated problem is the requirement of normal cells of the same cell lineage and comparable cell growth characteristics; these are not always available. When available, it is best to carry out the comparative studies of cancer and control cells at the same cell cycle phase, since growth-dependent changes of cellular glycopeptides have been documented (Buck *et al.*, 1971; Sakiyama and Burge, 1972; Muramatsu *et al.*, 1973).

## B. Metabolic Labeling of Cellular Glycoproteins

In this approach, a suitably labeled precursor sugar is added to culture medium containing metabolically active cells, organs, or tissue slices. The injection of isotopes into animals is uneconomical because the extensive dilution results in extremely poor incorporation into the desired tissue products. A number of precursor sugars can be utilized, the most commonly employed is glucosamine ([6-$^{3}$H] or [1-$^{14}$C]). This is the preferred sugar since all known animal glycoconjugates, with the exception of glycogen and collagen, contain at least one of the three sugars (*N*-acetylglucosamine, *N*-acetylgalactosamine, sialic acid) biosynthetically derived from it. Further, the major part of the added precursor is utilized in synthetic pathways (Kohn *et al.*, 1962). Recent studies in our laboratory, however, have shown that a significant proportion of glucosamine may be used by certain cultured cells for energy metabolism (O'Connor and Davidson, unpublished results).

L-Fucose is another precursor widely used for the metabolic labeling of glycoproteins. The use of this sugar was suggested by the pioneering studies of Bekesi and Winzler (1967) which indicated that it is exclusively incorporated as such into tissue glycoproteins and is not extensively metabolized. However, the subsequent isolation of various fucoglycolipids (Hakomori, 1975; McKibbin, 1978), fucose-containing glycosaminoglycans (Bray *et al.*, 1967), and fucose-free glycopeptides (Tai *et al.*, 1975; Bhavanandan *et al.*, 1977, 1981; Li and Kornfeld, 1979; Van den Eijnden *et al.*, 1979), and fucose-free glycoproteins (Spiro, 1973; Kawasaki and Ashwell, 1976a; Montreiul, 1980), does not support the earlier claim of exclusivity of fucose incorporation into glycoproteins. Nevertheless, this sugar as well as isotopically labeled mannose, galactose, and *N*-acetylmannosamine are useful for obtaining specific information on the carbohydrate composition and structure of cell surface glycoproteins (Yurchenco *et al.*, 1978). Metabolic labeling with a precursor sugar (for example, [6-$^{3}$H]glucosamine) and a precursor amino acid ([$^{14}$C]leucine) or [$^{35}$S]sulfate will help in subsequent purification since the second isotope serves to screen for nonglycosylated proteins or sulfated glycosaminoglycans, respectively.

## C. *In Vitro* Cell Surface Labeling of Glycoproteins

All of the carbohydrate moieties of the cell membrane glycoproteins (intrinsic and extrinsic) and portions of the protein are present on the outer surface of the intact cell. This arrangement makes it possible to isotopically label these glycoproteins by *in vitro* techniques and allows their

visualization after treating with carbohydrate-specific dyes, lectins, or antibodies (see Section VII,F).

Isotopic labeling of the exposed (available) sugar or amino acid on intact cells involves chemical modification using isotopic reagents under conditions that do minimal damage to the cells. The available methods include labeling of sialic acid residues by brief periodate treatment and tritiated borohydride reduction (Liao *et al.*, 1973; Gahmberg and Andersson, 1977) labeling of galactose residues by galactose oxidase oxidation followed by reduction with tritiated borohydride (Gahmberg and Hakomori, 1973) or coupling with methionine [$^{35}$S]sulfone hydrazide (Itaya *et al.*, 1975); labeling of available tyrosine by iodination with $^{125}I_2$ generated by treatment of Na$^{125}$I with lactoperoxidase and $H_2O_2$ (Phillips and Morrison, 1971; Hunt and Brown, 1975); and coupling of exposed $-NH_2$ (lysine) with pyridoxal phosphate to form a Schiff base which is stabilized by reduction with tritiated borohydride (Rifkin *et al.*, 1972). Labeling techniques including some not widely tested are summarized in Table II.

All of the above procedures attempt, not always successfully, to label only the surface-exposed molecules by employing either reagents that are too large (for example, lactoperoxidase, galactose oxidase) or highly charged (pyridoxal-5'-phosphate, formyl methionylsulfonemethyl phosphate) so that they do not penetrate the hydrophobic interior of the membrane. The exclusivity of these techniques has been questioned (Schmidt-Ullrich *et al.*, 1973; Juliano and Beher-Bennelier, 1975) but they are still suitable for preliminary studies. The periodate–borotritide labeling procedure was initially applied to erythrocytes (Liao *et al.*, 1973) and subsequently extended to lymphoid and other cell systems (Gahmberg and Andersson, 1977; Flowers and Glick, 1980; Brown *et al.*, 1981b). In our own preliminary work on various nucleated cultured cells (hepatoma, melanoma), it was found that 60–90% of the cells were permeable to Trypan Blue at the end of the labeling period (Bhavanandan, unpublished results).

It is thus clear that some of these techniques should be used judiciously and appropriate controls carried out, particularly if it is essential that *only* externally exposed residues are labeled. If it is critical to establish the surface localization of a glycoprotein, additional evidence should be obtained by independent criteria involving visualization techniques or by initial isolation of plasma membranes. Failure to label a molecule does not necessarily mean that it is absent at the surface since cryptic (sterically unavailable loci) molecules may not be acted upon by enzymes or other reagents. A serious disadvantage of the *in vitro* labeling technique is that glycoproteins adsorbed to the cell surface are not distinguished from true cell surface components. Strong adsorption of fetal calf serum components to the cell surface has been reported (Phillips and Perdue, 1977).

TABLE II
CELL SURFACE LABELING TECHNIQUES

| Reagents | Group(s) labeled | Reference |
|---|---|---|
| Sodium periodate and sodium [$^3$H]borohydride | Sialic acid | Liao *et al.* (1973); Gahmberg and Andersson (1977) |
| Galactose oxidase-sodium [$^3$H]borohydride | Galactose; *N*-acetylgalactosamine (preferentially nonreducing terminals) | Gahmberg and Hakomori (1973) |
| Lactoperoxidase–$H_2O_2$ or glucose oxidase and glucose and radioactive iodide | Tyrosine | Phillips and Morrison (1971); Hunt and Brown (1975) |
| Pyridoxal phosphate and sodium [$^3$H]borohydride | $-NH_2$ ($\epsilon NH_2$ group of lysine, aminoterminal) | Rifkin *et al.* (1972) |
| Galactose oxidase–methionine [$^{35}$S]sulfone hydrazide | Galactose, *N*-acetylgalactosamine | Itaya *et al.* (1975) |
| 4,4′-Diisothiocyano-2,2′-ditritiostilbene disulfonate ([$^3$H]DIDS) | $-NH_2$ | Cabantchik and Rothstein (1974) |
| [$^{35}$S]Formylmethionyl sulfone methyl phosphate ([$^{35}$S]FMMP) | $-NH_2$ | Bretscher (1971) |
| Diazobenzene [$^{35}$S]sulfonate (DABS) | $-NH_2$; tyrosine; histidine | Bender *et al.* (1971) |

Despite these limitations the *in vitro* labeling procedures, particularly the lactoperoxidase iodination and the galactose oxidase $-NaB^3H_4$ technique in conjugation with prior neuraminidase treatment, are very useful. At the present time, these techniques are of primary value in providing radioactive handles for the purification and preliminary characterization of minute quantities of glycoproteins (Fareed *et al.*, 1978). Improvements in application as well as development of newer methods can be expected to broaden the usefulness of this approach to the study of cell surface glycoproteins.

### D. ISOLATION AND PURIFICATION OF CELL SURFACE GLYCOPEPTIDES

Different strategies have been used for the isolation of cell surface glycopeptides of malignant cells. The two types discussed in this chapter are

those involving mild protease treatment of intact cells and exhaustive proteolysis of isolated plasma membranes.

*1. Mild Proteolysis of the Cell Surface Components*

Information on the nature of cell surface glycoproteins may be obtained by limited proteolysis of intact labeled or unlabeled cells resulting in controlled release of fragments. It is important to choose conditions of pH, ionic concentration of buffer, time, and temperature of incubation such that cell viability is not affected. Several investigators have used trypsin to release glycopeptides (and glycosaminoglycans) from the cell surface (Langley and Ambrose, 1967; Codington *et al.*, 1972; Saito *et al.*, 1977; Nakada and Yamashina, 1978; Debray and Montreuil, 1978). Codington *et al.* (1972) were able to release significant quantities of cell surface sialic acid in the form of glycopeptides from TA3 mammary adenocarcinoma cells. Using similar conditions, we were able to release mucin-type glycopeptides from B16 mouse melanoma cells metabolically labeled with [$^{14}$C]glucosamine (Bhavanandan *et al.*, 1977).

Walborg *et al.* (1969) and Robinson *et al.* (1976) were successful in solubilizing several glycoproteins from rat hepatoma cells by the use of papain. Papain seems capable of releasing larger glycoprotein fragments, which sometimes retain biological activity. Thus, biologically active antigens have been released from several cell types by treatment with papain (Shimada and Nathenson, 1969; Baldwin *et al.*, 1973; Viza and Phillips, 1975; Clemetson *et al.*, 1976; Ishii *et al.*, 1980). However since the conditions for maintaining cell viability are not optimal for papain activity, the yields of released glycopeptides are poor. Ficin (Langley and Ambrose, 1967) and bromelain are two other narrow spectrum proteases which may be useful for the release of cell surface components. In contrast to the above mentioned proteases, Pronase, which is a mixture of broad spectrum proteases, is generally not suitable for the release of surface components without affecting cell viability. The mildest conditions tried resulted in loss of viability of cultured cells (Bhavanandan, unpublished observations). However Ceccarini *et al.* (1975) and Ceccarini and Atkinson (1977) were able to isolate mannose-containing glycopeptides from monolayers of cultured human cells.

Mild proteolysis of intact cells probably results in the release of a selective rather than a representative population of surface glycopeptides. This is illustrated by the preferential release of epiglycanin (a peripheral glycoprotein) compared to other intrinsic membrane glycoproteins of TA3 cells. Further, this approach seems to work better on cells grown in suspension or Ascites form than on detached monolayer cultured cells since the latter have an already altered surface.

*2. Total Proteolytic Fragmentation of Isolated Membranes*

A majority of the studies on cell-associated glycoproteins have been carried out on limit protease (Pronase) digestion products of isolated plasma membranes (Meezan *et al.*, 1973; Glick and Buck, 1973; Nakada *et al.*, 1975; Warren *et al.*, 1978; Glick, 1979) or whole cells (Bhavanandan *et al.*, 1977, 1981; Muramatsu *et al.*, 1978; Li *et al.*, 1980; Takasaki *et al.*, 1980). This procedure has the advantage of yielding water-soluble fragments of membrane glycoproteins most of which have amphiphatic characteristics rendering them not readily soluble in aqueous systems. In addition, it is possible to obtain information on total carbohydrate changes at the cell surface without concern for the protein moiety; the latter is likely to influence solubilization, purification, and analytical procedures. Major disadvantages of this approach are that it does not provide information on changes of discrete glycoproteins and it is difficult to determine the relationship between observed differences and malignancy.

*3. Purification of Glycopeptides*

Glycopeptides derived from the proteolytic treatment of intact cells or plasma membranes can be broadly classified as illustrated in Fig. 1. Within each class, wide structural variations are possible; incompleteness of chains, mode and number of branches, linkage positions, etc. In addition to glycopeptides, glycosaminoglycans are present in digests of plasma membranes, a fact which has not always been previously considered. Several methods are available to separate glycosaminoglycans from glycopeptides. We have generally used cetyl pyridinium chloride to precipitate the glycosaminoglycans from the Pronase digests (Bhavanandan *et al.*, 1977, 1981; Chandrasekaran and Davidson, 1979). This procedure works well except for small losses of glycopeptides which occur during the precipitation of excess cetyl pyridinium chloride with KSCN followed by dialysis of the supernatant. An alternative technique, used in our studies on nuclear glycoconjugates, is fractionation by ion exchange chromatography on DEAE-cellulose or Sephadex columns (Bhavanandan and Kemper, unpublished results). This approach was only partially successful due to overlap of the elution of certain sialoglycopeptides and the non- or low-sulfated glycosaminoglycans (hyaluronic acid, chondroitin). If information on the nature of the glycosaminoglycans is not required, then it is possible to use enzymes and/or chemicals to selectively degrade these molecules. For example, hyaluronic acid and the chondroitin sulfates could be degraded by using hyaluronidases (Meyer, 1971) and chondroitinases (Suzuki, 1972), and the heparan sulfates by either heparitinase (Linker and Hovingh, 1972) or nitrous acid (Cifonelli, 1968). This ap-

proach has not been widely used apparently due to the difficulty in obtaining the requisite saccharases free of exoglycosidase activity. A recent study described the use of *Flavobacterium heparinum* culture filtrates to degrade chondroitin sulfates and heparan sulfates (Gill *et al.*, 1981).

Standard techniques such as gel filtration, ion exchange chromatography, and lectin affinity chromatography are then employed to fractionate the complex mixture of glycopeptides (Smith *et al.*, 1973; Bhavanandan *et al.*, 1977, 1981; Debray and Montreuil, 1978; Santer and Glick, 1979; Finne *et al.*, 1980a). In view of the intrinsic microheterogeneity of the oligosaccharide portion of glycopeptides and because the starting material consists of a pool of glycopeptides derived from a large number of individual glycoproteins, only mixtures of closely related glycopeptides rather than single species can be expected. For example, in work involving extensive purification, apparently homogeneous glycopeptides were obtained from metabolically labeled RSV-BHK cells (Santer and Glick, 1979) and HM7 human melanoma cells (Bhavanandan *et al.*, 1981). Structural investigation of these purified glycopeptides suggested reasonable homogeneity of the carbohydrate portions. However, it cannot be excluded that the isolated fractions contain glycopeptides of near-identical carbohydrate composition and array derived from several different glycoproteins or different areas of the same glycoproteins. A corollary to this is that amino terminal analysis cannot be used as a purity criterion for glycopeptides.

### E. Isolation of Intact Cell Surface Glycoproteins

To understand the relationship between cell surface glycoproteins and malignancy, it will be essential to isolate individual components, ideally, with retention of biological activity. Membrane glycoproteins can be grouped into two categories: peripheral or extrinsic glycoproteins which interact minimally with other membrane components and integral or intrinsic glycoproteins which are partly buried in the bilayer. The possibility of extracting the extrinsic glycoproteins without damaging the intact cell has not been fully investigated. The only extrinsic protein extracted in this manner is cellular fibronectin (LETS glycoprotein) which was solubilized by treatment of cell monolayers with urea (Yamada and Weston, 1974).

The solubilization of intrinsic glycoproteins presents technical problems due to the partial hydrophobic nature of these molecules. In general, these amphipathic glycoproteins can be extracted only after prior purification of plasma membranes. The membranes are then treated with deter-

gents to disrupt the lipid bilayer in essence substituting the hydrophobic lipid surrounding the glycoprotein by hydrophobic detergents.

### *1. Plasma Membrane Isolation*

A variety of techniques are available for the isolation of plasma membranes; as a rule, procedures are continually being improved in terms of speed, yield, and purity (Steck and Wallach, 1970; Brunette and Till, 1971; Warren, 1974; Perdue, 1974; Neville, 1976). Some of these methods have been adapted for zonal centrifugation and therefore can be used for large scale preparations. A rapid method for the isolation of surface membranes from tissue culture cells involves cell lysis in hypoosomotic borate buffer–EDTA followed by differential centrifugation (Thom *et al.*, 1977). The method of Barland and Schroeder (1970) involving the stripping of membrane from monolayer cells with fluorescein mercuric acetate is rapid and simple but may result in selective loss of some membrane components (Quissel *et al.*, 1977). Two novel methods described for the isolation of erythrocyte plasma membranes involve (1) the use of polylysine coupled to glass beads (Jacobson *et al.*, 1978) and (2) the introduction of polydeoxythymidylic acid chains to the membrane followed by hybridization to polyadenylic acid–agarose (Wennogle and Berg, 1978). The applicability of these methods to other cell membranes has not been reported.

### *2. Solubilization of Plasma Membranes*

Prior to solubilization of the glycoprotein, extraction of the membranes with chloroform–methanol mixtures helps to remove lipids and glycolipids (Hakomori and Murakami, 1968). If this is done, then consideration should be given to the possibility that certain glycoproteins may partition, in part, in the organic phase (Watanabe *et al.*, 1980). This could be due to either an overall hydrophobic nature or to strong binding of lipids. This treatment is likely to denature many membrane glycoproteins resulting in the loss of biological activity, if any.

The glycoproteins that are interacting with the lipid bilayer by electrostatic interactions may be solubilized with suitable changes in the ionic strength and/or pH of the extracting medium. Sodium chloride of varying molarity has been used to extract membrane proteins (Rosenberg and Guidotti, 1969; Fairbanks *et al.*, 1971; Braatz *et al.*, 1978). In these and other similar extractions involving 3 *M* KCl (Reisfeld *et al.*, 1971; Leonard *et al.*, 1975; Chee *et al.*, 1976) the role of endogenous proteases should be considered since some activation by salt solutions appears to occur (Mann, 1972). Therefore, if undegraded glycoproteins are to be obtained, it is essential to include protease inhibitors such a toluene sulfonyl fluo-

ride, diisopropyl fluorophosphate, pepstatin, aprotinin, and iodoacetamide in the extracting medium. Extraction of whole cells with 3 *M* KCl gave active histocompatibility antigens (Reisfeld *et al.*, 1971) and active melanoma-associated antigen (Bhavanandan *et al.*, 1980) indicating that limited autoproteolysis may not always be undesirable. Treatment with chelating agents such as EDTA (Wise *et al.*, 1975; Dunn *et al.*, 1975) or with the hydrogen-bond breaking agents, urea (Tanner and Gray, 1971) or guanidine hydrochloride (Gwynne and Tanford, 1970), are other methods for extracting extrinsic membrane proteins. If high concentrations (4–8 *M*) of urea or guanidine HCl are used, the effect of the high density of these solutions should be taken into account in evaluating results. Protein–lipid complexes are small vesicles and are likely to float during centrifugation of the extracts giving the false impression of effective solubilization (Maddy and Dunn, 1976).

Solubilization of intrinsic membrane glycoproteins requires the use of chaotropic agents (potassium or sodium thiocyanate, lithium diiodosalicylate) or organic solvents (butanol, pyridine, phenol) or detergents. Lithium diiodosalicylate introduced by Marchesi and Andrews (1971) for the purification of glycophorin from human erythrocyte membranes has been successfully used for the extraction of glycoproteins from plasma membranes of AH66 rat hepatoma (Funakoshi and Yamashina, 1976; Nakajo *et al.*, 1979) of BHK cells (Tuszynski *et al.*, 1978) and HM7 human melanoma (Umemoto *et al.*, 1981). The strong affinity of lithium diiodosalicylate to glycophorin and probably other membrane glycoprotein was demonstrated by Segrest *et al.* (1979) who recommended the use of sodium deoxycholate for the purification of glycophorin. Butanol, pyridine, and phenol have been used by several investigators to solubilize erythrocyte ghosts (Maddy, 1966; Zwaal and van Deenan, 1968; Blumenfeld and Zvilichovsky, 1972; Howe *et al.*, 1972) but have not been applied to other animal cell membranes. Extraction with chloroform–methanol to partition the lipid and protein components of the erythrocyte membrane used by Hamaguchi and Cleve (1972) was recently adapted for the extraction of glycoproteins from thyroid plasma membranes (Okada and Spiro, 1980).

The most popular agents for solubilizing membranes are detergents. Both nonionic (Triton X-100, Nonidet P-40, octyl glucoside, Triton CF-54, Tween 20) and ionic (sodium deoxycholate, sodium dodecyl sulfate, sodium dodecyl sarcosinate) detergents have been used (Maddy and Dunn, 1976). In general, the former category is preferable since these appear to cause the least alterations in the structure and conformation of the glycoproteins as illustrated by the absence of their influence on the activities of antibodies (Dimitriadis, 1979) and lectins (Lotan *et al.*, 1977) at concentrations of about 1%. These detergents can also be expected to

have minimal effect on enzyme and receptor activities. In contrast, the ionic detergents bind strongly to proteins (Reynolds and Tanfold, 1970) and can thus cause irreversible conformational changes. They also interfere in the interaction of antibodies and lectins with glycoproteins (Lotan *et al.*, 1977; Dimitriadis, 1979). Although the nonionic detergents have obvious advantages, the use of ionic detergents is still valuable under special circumstances because of their ability to cause complete solubilization of most membrane glycoproteins.

Once the proteins are solubilized the detergents may, in some instances, be removed without causing precipitation. For example, glycophorin remains in aqueous solution after removal of detergents, probably due to its high carbohydrate content. However, in most cases this procedure is likely to cause aggregation or insolubilization of the glycoproteins (Kawasaki and Ashwell, 1976b; Dorst and Schubert, 1970; Bhavanandan *et al.*, 1980). Thus, an optimum concentration of the detergent should be maintained throughout purification and subsequent studies to ensure that the component of interest remains in solution. The nonionic detergents are usually removed by extensive dialysis, treatment with resins such as BioRad Sm-2 (Holloway, 1973), or by extraction with ice-cold ethanol, or acetone (Funakoshi and Yamashina, 1976; Kawasaki and Ashwell, 1976b). The removal of unbound detergents from proteins is discussed in a recent review (Furth, 1980).

*3. Fractionation of the Solubilized Gycoproteins*

Generally it is advisable to include protease inhibitors in the buffers during purification procedures. The direct and simplest method of purifying a cell surface glycoprotein is by affinity chromatography on an antibody–Sepharose column, a technique obviously dependent on the availability of an appropriate antibody directed against the glycoprotein of interest. In view of recent advances in the production of monoclonal antibodies, future widespread use of this method can be expected (Goding, 1980; Schlom *et al.*, 1980; Mitchell *et al.*, 1980; St. Groth and Scheidegger, 1980). Several investigators have successfully employed monoclonal antibodies for the purification of cell surface glycoproteins. Thus, Brown *et al.* (1981b) isolated a glycophorin-like molecule from rat thymocytes; Hellstrom and co-workers (Yeh *et al.*, 1979; Woodbury *et al.*, 1980) isolated a glycoprotein (P97) from human melanoma cells and Momoi *et al.* (1980) isolated a membrane glycoprotein from human neuroblastoma cells by this technique.

Affinity chromatography on lectin columns is another useful technique for purifying membrane glycoproteins. Because of the wide carbohydrate specificities of lectins such as concanavalin A, wheat germ agglutinin, and

the *Ricinus communis* agglutinins, affinity chromatography using these lectins will result in the separation of classes of glycoproteins rather than individual components. If the glycoprotein under investigation binds to an immobilized lectin, then this property can be used successfully to remove all nonglycosylated proteins as well as the nonbinding glycoproteins. In some cases, a specific glycoprotein may entirely bind to a lectin column, as in the case of 5′-nucleotidase of pig lymphocyte membrane (Hayman and Crumpton, 1972). In other cases, it is possible that portions of the same glycoprotein may bind incompletely to one lectin or to different lectin columns due to carbohydrate heterogeneity. Subfractionation of the class of glycoproteins binding to a particular immobilized lectin may be possible by gradient elution with appropriate sugars. In the case of wheat germ agglutinin, subfractionation should also be possible by using a series of columns packed with lectin–Sepharose of varying concentration of lectin per milliliter gel (Bhavanandan and Katlic, 1979).

A note of caution must be introduced regarding the use of affinity chromatography as a structural tool. The characteristics of affinity chromatographic behavior will indicate only general structural information and, within that restriction, only those saccharide moieties which are accessible to and recognized by the affinity ligand. Affinity will also differ depending on the nature and preparation of the glycopeptides. For example, it is quite likely that a fragment with two saccharide units will have quite different behavior on a given lectin affinity column than one with a single saccharide unit. Furthermore, the density of the lectin on the supporting matrix is a critical factor in evaluating chromatographic behavior. Thus, comparisons from one laboratory to another, even with identical cell systems, are very difficult to make.

The nominal structural basis for lectin specificities are often inaccurate. Adsorption to and elution from a particular lectin column with a haptenic monosaccharide eluent (for example utilization of *N*-acetylglucosamine with wheat germ agglutinin–Sepharose columns) does not at all mean that such sugars are present in the glycoprotein under investigation (Bhavanandan and Katlic, 1979). This kind of oversimplification is frequently encountered and can lead to mistaken identification of saccharide residues on glycoproteins. Most lectins have specificities which either encompass oligosaccharide units or have sufficiently broad specificities that saccharides of more than one structure can be accommodated within the binding site. Nonetheless, with these caveats in mind, the profile of interaction of glycopetides or glycoproteins derived from given cell lines can be quite revealing and indicate changes in accessible carbohydrate associated with transformation.

Due to this lack of strict specificity of most lectins and the inherent mi-

croheterogeneity of the oligosaccharides of glycoproteins, it is essential to first test the lectin columns used for purification with glycoproteins and glycopeptides of known structures. In addition it is also important to assess the yield of the glycoprotein from lectin affinity steps because irreversible interaction between the lectin and the ligand is known to occur. Affinity chromatography on immobilized antibody or lectin columns can usually be performed in the presence of a number of detergents (Lotan *et al.*, 1977; Lotan and Nicolson, 1979).

Purification of cell surface glycoproteins can also be carried out by a combination of conventional techniques. Gel filtration on several supports (Sepharose CL, Sephacryl, Controlled Pore Glass beads) can be carried out in the presence of nonionic detergents (Triton X-100; Ammonyx; Nonidet P-40), ionic detergent (sodium dodecyl sulfate), or hydrogen bond breaking solvents (urea, guanidine–HCl). It is necessary, however, to determine the influence (interference) of the buffer additives on the spectrophotometric, colorimetric or isotope counting procedures used for analysis of the fractions. Ion exchange chromatography on DEAE- or CM-Sepharose is usually carried out by gradient elutions which NaCl or LiCl in buffers containing nonionic detergents. Volatile buffers such as pyridinium acetate, widely used for the fractionation of glycopeptides, are not suitable for membrane glycoproteins because of their poor solubility. The purification of mouse melanoma-associated antigen (Bhavanandan *et al.*, 1980) and a 100K glycoprotein from human melanoma cells (Umemoto *et al.*, 1981) was accomplished by repeated ion exchange chromatography on DEAE-Sepharose columns using buffers containing 0.1% Triton X-100. Isoelectric focusing in neutral detergents and urea (Merz *et al.*, 1972; Zechel, 1977; Baumann and Doyle, 1979) has also been used for purification of glycoproteins. A limitation of this method is that the high sensitivity usually results in separation based on microheterogeneity of the oligosaccharides. Thus, a given glycoprotein may given rise to several peaks based on single differences in sialic acid or other charged residues. Preparative polyacrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate has also been used for the purification of glycoproteins even though the recoveries in such experiments are usually low (Baldwin *et al.*, 1973; Dunn *et al.*, 1975; Braatz *et al.*, 1978).

### F. Mapping the Organization of Cell Surface Glycoproteins

An evaluation of the role of cell surface glycoproteins in malignancy requires more than information on individual glycoproteins. It is possible that the altered properties of cancer cells such as proliferative growth,

metastasis, altered recognition, and adhesion may not be due to changes in individual cell surface components but rather to a composite influence of several "new" components. Thus any study on cell surface glycoprotein should also assess the supramolecular organization of these and of other related molecules (glycolipids and glycosaminoglycans). Useful tools for these studies are (1) specific chemical stains, (2) lectins of well-defined specificities, (3) antibodies produced against known oligosaccharide structures, and (4) highly purified exo- and endoglycosidases. The gross distribution of cell surface carbohydrates can be determined by the help of light or electron microscopy after treatment of the cells with the first three classes of the above reagents.

### *1. Studies with Chemical Stains*

Typical chemical reagents used include periodate–Schiff (Rambourg *et al.*, 1966; Ito, 1969; Martinez-Palamo, 1970; Luft, 1976), ruthenium red (Vorbradt and Koprowski, 1969; Luft, 1971), and colloidal iron (Hale, 1946; Gasic and Berwick, 1963; Rambourg *et al.*, 1966; Benedetti and Emmelot, 1967). Treatment of the intact cells with enzymes (neuraminidase, hyaluronidase) as well as carrying out the staining reactions at varying pH would help to distinguish between glycosaminoglycans and sialoglycoproteins or sialoglycolipids (Gasic and Berwick, 1963; Huet and Herzberg, 1973; Kim *et al.*, 1975). For example, at pH 2.0 only about 10% of the sialic acid carboxyl is ionized whereas sulfate ester groups are fully dissociated.

### *2. Studies with Lectins*

Lectins are proteins (or glycoproteins) from plant or other sources which can bind noncovalently to specific carbohydrate groups but are known not to be enzymes, transport proteins, or immunoglobulins. Because of the abundance of carbohydrates on the cell surface (glycocalyx), the multivalent lectins are able to cause agglutination of erythrocytes and other cells, sometimes after prior enzymatically catalyzed modification of the cells. The findings that wheat germ agglutinin caused differential agglutination of normal and transformed cells stimulated a great deal of research on lectins (Aub *et al.*, 1965; Burger and Goldberg, 1967). Subsequently several other lectins (concanavalin A, soybean agglutinin, *Ricinus communis* agglutinins) were found to agglutinate cancer cells at much lower concentrations than control (normal) cells (Brown and Hunt, 1978). It was also noted that mild proteolysis rendered normal cells as agglutinable as transformed cells (Inbar *et al.*, 1969).

Lectins have been used in several additional ways to explore cellular

glycoconjugates. The binding of lectins to cell surfaces can be quantitated by the use of isotopically labeled lectins (see reviews by Nicolson, 1974, 1976), preferably the monovalent subunits such as succinylated Con A (Gunther *et al.*, 1973). The topographical distribution of sugars can also be assessed by one of several methods: (1) direct immunofluorescence using lectin conjugated to fluorescent dyes such as fluorescein isothiocyanate or rhodamine (Inbar and Sachs, 1973); (2) indirect immunofluorescence using lectin and a fluorescein-conjugated antibody to the lectin (Mallucci, 1971); (3) electron microscopic visualization of lectins conjugated with ferritin (Nicolson and Singer, 1971, 1974; Marchesi *et al.*, 1972); (4) electron or light microscopic autoradiography using isotopically labeled lectins (Rapin and Burger, 1974); (5) histochemical studies with covalent conjugates of lectins and peroxidase (Bernhard and Avramaes, 1971; Huet and Garrido, 1972; Martinez-Paloma *et al.*, 1972).

### *3. Studies Using Antibodies against Oligosaccharides*

Because most lectins lack strict or defined saccharide specificity other, better defined tools are desired. Antibodies directed against known oligosaccharides have great potential since they can be used to probe cell surface glycoconjugate organization in the same manner as that discussed above for lectins. Antibodies against neuramin 2→3 and 2→6 lactose and other sialyloligosaccharides (Smith and Ginsburg, 1980); lacto-*N*-fucopentaose III, lacto-*N*-tetraose, and mannotetraose (Zopf *et al.*, 1975, 1978); galactose and lactose (Pazur *et al.*, 1978); galactose and Lewis-a-b and -d determinants (Lemieux *et al.*, 1981) have all been prepared. Antibodies against specific oligosaccharides of both the *N*-glycosidically and *O*-glycosidically linked types known to be present on normal and transformed cells can be expected as a result of the rapidly expanding technology of monoclonal antibody.

### *4. Studies Using Glycosidases*

The use of glycosidases to probe the cell surface distribution of saccharides has been largely confined to the use of sialidases (Hughes *et al.*, 1972; Barton and Rosenberg, 1973; Simmons and Rios, 1973; Weiss *et al.*, 1974). The availability of other highly purified exo- and endoglycosidases of known substrate specificities which are active at near physiological pH and ionic conditions should make further studies possible. The applicability of this approach is also limited since most of the presently available glycosidases do not effectively hydrolyze high-molecular-weight glycoprotein substrates (Flowers and Sharon, 1979). The endo $\beta$-*N*-acetylglucosaminidases (Muramatsu, 1978; Tarentino *et al.*, 1978), endo-$\beta$-galacto-

sidases (Takasaki and Kobata, 1976; Nakagawa *et al.*, 1980), a glycopeptidase acting on aspartylglycosylamine linkages (Takahashi and Nishibe, 1978, 1981), and endo-$\alpha$-*N*-acetylgalactosaminidase (Umemoto *et al.*, 1977; Glasgow *et al.*, 1977) are enzymes which satisfy some of the above requirements (Fukuda *et al.*, 1979).

It is important to consider the effect of the cell surface glycosaminoglycans in these studies. The results of experiments on cells before and after treatment with hylauronidase, chondroitinase (Nakada *et al.*, 1977), and heparitinase should be compared. The degradation of cell surface heparan sulfate by heparitinase was recently reported (Ohkubo *et al.*, 1981).

## G. Structural Analysis of the Isolated Glycopeptides and Glycoproteins

Before embarking on structural studies, it is essential to establish homogeneity of the component under investigation. This has proved difficult because of the heterogeneous nature of the carbohydrate chains (Horowitz, 1977). In the case of homogeneous preparations of nonglycosylated proteins, techniques such as gel filtration, ion-exchange chromatography, gel electrophoresis, and isoelectric focusing can be expected to give single sharp symmetrical peaks or single sharp bands. In contrast, a pure preparation of glycoprotein, because of variations in the intra- and intermolecular carbohydrates, cannot be expected to yield similar results. An additional complication in the case of membrane glycoproteins is the ability of these molecules to aggregate resulting in either discrete bands as in the case of glycophorin (Silverberg and Marchesi, 1978) or a broad spectrum of aggregates (Kawasaki and Ashwell, 1976b; Dorst and Schubert, 1979; Bhavanandan *et al.*, 1980). This difficulty might be overcome by determining the homogeneity of the protein which carries the saccharide chains. If sufficient (microgram) quantities are available, $NH_2$ or COOH terminal amino acid determination may provide this information.

A second approach which can be used at tracer levels provided the protein is isotopically labeled (either *in vivo* for example, [$^{14}$C]leucine or *in vitro* by reductive methylation with H$^{14}$CHO) is chemical or enzymatic deglycosylation followed by examination of the resultant polypeptide. Unfortunately, this method is not widely applicable since complete enzymatic deglycosylation with the available exo- and endoglycosidases can be seldom achieved. The applicability of chemical deglycosylation with hydrogen fluoride (Mort and Lamport, 1977; Glassman *et al.*, 1978) as a general method for all glycoproteins has yet to be established.

Structural studies are usually not carried out on intact glycoproteins. The presence of more than one type of carbohydrate chain on the same

molecule makes it difficult to interpret the results and the large quantity of amino acid interferes in many of the analytical procedures. Thus, glycoproteins are usually subjected to proteolysis with broad spectrum proteases such as Pronase and the products fractionated by a combination of techniques (gel filtration, ion exchange chromatography, lectin affinity chromatography, etc.) to yield individual, homogeneous glycopeptides. The few amino acids present on Pronase generated glycopeptides minimally influence fractionation. This is in contrast to differences in saccharide composition, where a difference of just one sugar particularly if it is charged, has a significant effect on chromatographic and electrophoretic properties. The aim of the fractionation approach should be to separate the different classes of glycopeptides (Fig. 1) rather than to separate on the basis of minor variations. Lectin affinity chromatography appears to be specifically useful for this purpose (Ogata *et al.*, 1975; Krusius *et al.*, 1976; Narasimhan *et al.*, 1979; Rasilo, 1980; Bhavanandan *et al.*, 1981).

*1. Isolation of Peptide-Free Oligosaccharides*

An alternative approach, for the determination of carbohydrate structure of glycoproteins involves the release of all carbohydrate chains from the protein thus totally eliminating the influence of the protein (or peptide) during fractionation (Finne *et al.*, 1980a). The mild alkaline–borohydride treatment conditions established for the release of *O*-glycosidically linked saccharides with minimum degradation (Carlson, 1968; Spiro, 1972) have been used extensively for this purpose. The release of the *N*-glycosidically linked saccharides without destruction is best achieved by hydrazinolysis (Yosizawa *et al.*, 1966). The use of this technique for structural elucidation has recently been reported by several investigators (Fukuda *et al.*, 1976; Reading *et al.*, 1978; Takasaki *et al.*, 1980; Yoshima *et al.*, 1980). The degradation of the oligosaccharides thus released appears to be minimal provided strictly anhydrous conditions are used. Strong alkaline–borohydride treatment recommended for the release of such carbohydrate chains (Lee and Scocca, 1972) was found to cause extensive degradation of the released saccharides of $\alpha_1$-acid glycoprotein and fetuin (Bhavanadan, unpublished results). On a limited scale, endoglycosidases can be used to release oligosaccharides from glycoproteins (Flowers and Sharon, 1979; Kobata, 1979).

The oligosaccharides derived from chemical or enzymatic cleavage are then fractionated into different classes. This can be done by gel filtration on fine and superfine gels (Etchison *et al.*, 1977; Van den Eijnden *et al.*, 1979; Natowicz and Baenziger, 1980) ion exchange chromatography (Finne, 1975; Fukuda *et al.*, 1976), thin-layer chromatography (Holmes and O'Brien, 1979), paper chromatography (Bhavanandan *et al.*, 1977,

1981; Fukuda and Hakomori, 1979; Li and Kornfeld, 1979), high voltage paper electrophoresis (Li and Kornfeld, 1979; Takasaki *et al.*, 1980; Yoshima *et al.*, 1980), high-pressure liquid chromatography (Wells and Lester, 1979), and lectin affinity chromatography (Blake and Goldstein, 1980). Reduction of the oligosaccharide with sodium borotritide facilitates the fractionation and analysis of trace amounts.

Once homogeneous preparations of glycopeptides or oligosaccharides are available, one can proceed with the determination of the following: (1) the nature of linkage of carbohydrate to peptide, (2) the sugar composition, (3) the sequence of the sugars, (4) the anomeric configuration of each of the glycosidic bonds, (5) the linkage positions, that is, which hydroxyl groups other than the glycosidic hydroxyls are involved in the linkages. The details of the methods used to obtain this information can be found in prior reviews (Spiro, 1972; Marshall and Neuberger, 1972a; Montreuil, 1975). Thus, only a brief description of the approach is given below.

### 2. *Nature of Carbohydrate–Peptide Linkage*

This information can be obtained by mild alkaline treatment in the presence of borohydride, hydrazinolysis, and by the use of glycosidases. The $\beta$-elimination reaction with mild alkali, however, is very slow if the glycosylated amino acid is either $NH_2$ or COOH terminal and can be made more efficient by prior blocking of these groups (Neuberger *et al.*, 1972). $\beta$-Elimination in the presence of sodium borohydride in addition to identifying *O*-glycosidically linked oligosaccharides also permits the determination of the proportion of serine and threonine substituted by estimating the increase in alanine and appearance of $\alpha$-aminobutyric acid. Further, by carrying out the elimination reaction in the presence of sodium sulfite instead of sodium borohydride it is possible to convert the hydroxyamino acids involved in the linkage to sugar to cysteic acid and $\alpha$-amino-$\beta$-sulfonylbutyric acid (Harbon *et al.*, 1968; Simpson *et al.*, 1972; Spiro and Bhoyroo, 1974). Use of $Na^{35}SO_3$ and sequencing of the peptides would provide information on the location of the sugar-linked hydroxy amino acid in the protein (Simpson *et al.*, 1972; Isemura and Ikenaka, 1975).

### 3. *Determination of Sugar Composition*

Several colorimetric methods, including modifications for microdeterminations, are available for quantitating hexoses, hexosamines, deoxyhexoses, and sialic acid (Ashwell, 1966; Spiro, 1966a; Marshall and Neuberger, 1972b). The currently preferred approach however, is gas liquid chromatographic analysis which is more sensitive and rapid. A very useful procedure is methanolysis of the glycopeptide or oligosaccharide fol-

lowed by trimethylsilylation of the methyl glycosides; this permits determination in one analysis of the total sugar composition (Clamp *et al.*, 1971; Laine *et al.*, 1972; Clamp, 1977). Trifluoroacetylation of methyl glycosides, instead of trimethylsilylation (Montreuil, 1975) is a good alternative since the use of electron capture detection would increase the sensitivity of the analysis several fold (Pritchard and Niedermeier, 1978). However, this procedure has not been widely tested. Alternatively one may analyze the sugars (except sialic acid) as alditol acetates (Lehnhardt and Winzler, 1968; Sloneker, 1972; *et al.*, 1972). Another technique with good potential which needs further development is high-pressure liquid chromatographic separation of underivatized sugars following acid hydrolysis (Tikhomirov *et al.*, 1978; Binder, 1980). This method would permit the recovery of the sugars for further analysis and confirmation of identify. For identification and quantitation of hexosamines the amino acid analyzer has been widely used. The micro amino acid analyzers presently available make it possible to analyze picomole quantities of hexosamines present in hydrolysates of glycopeptides and glycoproteins.

These chromatographic procedures can be adapted to quantitate isotopically labeled sugars by stream-splitting the column effluent and collection of fractions for scintillation counting. Prehm and Scheid (1978) were able to quantitate 0.2 nmoles of monosaccharide by gas liquid chromatography of $^{3}H$-labeled alditol acetates. We have made extensive use of this technique in conjunction with the amino acid analyzer to quantitate the distribution of label in glucosamine and galactosamine (Bhavanandan and Davidson, 1976; Bhavanandan *et al.*, 1977, 1981).

Several other techniques for the determination of trace amounts of sugars have been published (Takasaki and Kobata, 1974; Knutson, 1975; Roll and Conrad, 1977; Hase *et al.*, 1978; Hara *et al.*, 1979; Barr and Nordin, 1980).

*4. Determination of Structure*

There is no single method available that will provide information on the sequence, anomeric configuration, and linkages of sugars in glycoproteins and glycopeptides. The three widely used techniques are methylation analysis (Lindberg, 1972; Montreuil, 1975), treatment with glycosidases and periodate oxidation (Smith Degradation) (Hay *et al.*, 1965; Goldstein *et al.*, 1965). The enzymatic method provides the most information since all glycosidases have sugar and anomeric specificity and in addition certain glycosidases also have linkage and aglycon specificity. Thus it is possible to distinguish (a) between Gal$\beta$1 $\rightarrow$ 4GlcNAc and Gal$\beta$1 $\rightarrow$ 3GlcNAc and Gal$\beta$1 $\rightarrow$ 6GlcNAc by using the appropriate $\beta$-galactosidase (Li *et al.*, 1975; Paulson *et al.*, 1978), (b) between man$\alpha$1 $\rightarrow$ 2 man,

man$\alpha$1 → 3 man, and man$\alpha$1 → 6 man using *Aspergillus saitei* $\alpha$-mannosidase (Yamashita *et al.*, 1980), and (c) between NeuNAc 2 → 3 Gal and NeuNAc 2 → 6 Gal using influenza virus neuraminidase (Drzeniek, 1967; Bhavanandan *et al.*, 1977). It can be hoped that the discovery of additional glycosidases (particularly from mammalian sources), the elucidation of the exact specificity of the existing and newly described glycosidases, and their ready availability would widen the use of this approach. For information on currently available glycosidases and their use in structural elucidation, readers are referred to recent reviews (Li and Li, 1977; Flowers and Sharon, 1979; Kobata, 1979).

At present, the most productive approach for structure elucidation is the combination of sequential degradation of the sugar chain from the nonreducing end with glycosidases and methylation (Hakomori, 1964) of the residual portion. This is followed by total hydrolysis and identification of the methylated sugars by GC/MS analysis (Bjorndal *et al.*, 1970). The application of this has been described in several recent studies (Kornfeld, 1978; Fukuda and Hakomori, 1979; van den Eijnden *et al.*, 1979; Finne *et al.*, 1980b; Irimura *et al.*, 1981).

Periodate oxidation is useful in obtaining information on linkage position, branching (Goldstein *et al.*, 1965; Spiro, 1966b), and the substitution position of *N*-acetyl hexosaminitols (Spiro, 1972; van den Eijnden *et al.*, 1976). Because of the differences in the rate of hydrolysis of different glycosidic bonds and of different sugars, partial degradation of carbohydrate chains can be achieved by mild acid hydrolysis (Wolfrom and Franks, 1965; Painter, 1965), acetolysis (Bayard and Montreuil, 1972; Bayard *et al.*, 1975), or hydrazinolysis followed by nitrous acid treatment (Isemura and Schmid, 1971; Montreuil, 1975; Aspinall *et al.*, 1980; Strecker *et al.*, 1981). The resulting fragments can then be sequenced and based on overlapping fragments the structure of the complete carbohydrate chain reduced.

A technique which is being increasingly applied to structural elucidation of saccharides is high resolution (360 to 500 MHz) $^1$H NMR spectroscopy, as illustrated by the studies of Fournet *et al.* (1978) and Montreuil and Vliegenthart (1979).

## VIII. General Recapitulation

Insofar as generalizations can be made regarding the presence of unusual glycoproteins on the surfaces of transformed cells, it appears that such are likely to be found. The most common properties appear to be related to both increased complexity and increased glycosylation of the saccharide moieties of the glycoproteins. Studies conducted by utilization

of tritiated sugar precursors or by labeling of saccharide residues in whole cells or membrane preparations followed by glycopeptide isolation have supported the generality of these conclusions. What is much less clear is the tissue-specific origin of the polypeptide portion of these macromolecules. Recent work, which is rather heavily dependent on modern immune technology (production of monoclonal antibodies by the hybridoma technique), has provided strong suggestive evidence showing that the tissue-specific antigen is present in all tumors of a particular type or that it is not present in a variety of tumors of another cell type, as well as adult or fetal cells. One difficulty with the immune technique is that alterations in the carbohydrate moiety of the glycoprotein can easily result in sufficient conformational changes to evoke new antigenic determinants from common polypeptide precursors. Thus, even the sophisticated hybridoma technology may be led astray in presuming that totally new structures are being formed. Without the essential chemical evidence regarding the primary structure of glycoprotein products, it will be impossible to define them as unique and specific products of tumor cell metabolism. It is quite likely that the transformation event results in expression of new genes but it may also result in the modification of preexisting membrane associated glycoproteins and their subsequent appearance as new bands on gels or new antigens in immune screening procedures.

Modern genetic technology would presume that appearance of new phenotypic characteristics would, if associated with a new genotype, be detectable by appropriate techniques. Thus, the presence of a new messenger RNA could be detected if an appropriate library of cloned fragments were available for the necessary hybridization studies. It should be noted that most of the surface components mentioned are present in extremely small quantities, have been detected primarily if not exclusively by isotopic tracer techniques, and the prospect of fishing out the appropriate genetic information must be accorded as relatively remote.

A second problem in dealing with tumor-related cell surface glycoproteins involves their length of residence on the cell surface. It has been reported by a number of laboratories that transformed cells exhibit an elevated level of surface protease activity. This may well account for the apparent loss of fibronectin from the surface of fibroblasts following viral transformation. The altered behavior of the cell surface glycoproteins may arise from the fact that localized processing of intrinsic membrane proteins can occur under circumstances where this event would not have taken place prior to transformation. Furthermore, the putative proteolytic activity could be responsible for the loss from the cell surface of membrane components at a much higher rate (observations confirmed by direct experimentation) than is found in normal cells. As a result of this,

increased levels of membrane proteins may appear in the circulation. At least one report has appeared of a circulating glycoprotein associated with malignancy although its identification with the cell surface has not been made (Bolmer and Davidson, 1981). The generality of this observation, that is, the association of this glycoprotein with a variety of malignancies, suggests that origin within a specific tumor cell is less likely than is modification of a preexisting component by a common feature associated with cells that have lost growth control.

## IX. Prognosis

The general trend in recent years has indicated that a very large variety of tumor-related glycoprotein antigens will continue to be described. Most of the descriptive material will rely on either metabolic or surface labeling approaches followed by identification of unusual components by gel electrophoretic or immunologic techniques. Sophisticated refinement such as the use of two-dimensional gels or isoelectric focusing gels and monoclonal antibodies are likely to improve the number of qualitative changes that are identifiable but are not likely to provide an understanding of the nature of these differences or the relationship between the characteristics of the transformed cell and the expression of the unusual component. By quite a different route, descriptive material will continue to be acquired based largely on techniques such as lectin affinity chromatography and electron microscopic visualization of surface features utilizing either lectin or other probes derivatized to provide localization information. Here, differences may well be established, but their chemical nature will be very difficult to interpret. Thus, changes in lectin affinity chromatographic profiles can arise from alterations in the carbohydrate composition of glycoproteins or loss of one or another component from the cell surface, and so on. The relationship of these observations to the more explicit ones derived by direct biochemical techniques, will rarely be accomplished in the same laboratories. A key problem that remains has been alluded to above and relates to the explicit chemical definition of observed alterations. It is necessary to isolate sufficient material from any system to define the chemistry and the origin of the macromolecule involved. The next step will be to associate these changes with specific gene expression and to document the generality of such a change with the transformed phenotype. The latter is perhaps the most difficult problem and its solution one that has thus far eluded researchers. The fact remains that the fundamental biologic problem in malignancy has yet to be defined in spite of, for example, a complete knowledge of the gene architecture of

a transforming virus. Those events which result in loss of growth control, perhaps the initial problem, followed by selection from within that population of cells that have the ability to metastasize, the life threatening event, is still far from understood. The best information that is likely to derive from continued studies on cell surface glycoproteins has to do with either diagnostic or immunologic approaches to the management of the disease.

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Reprinted from Biochemistry, 1982, *21*, 694


# Isolation, Purification, and Properties of Respiratory Mucus Glycoproteins†

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ABSTRACT: The major glycoprotein from human tracheobronchial secretions and from primary explant cultures of human tracheal epithelium has been purified to apparent homogeneity. Mucin was solubilized in buffer and fractionated on Sepharose CL-4B, followed by CsBr density gradient centrifugation of the void volume fraction. High- and low-density fractions were obtained in ratios ranging from 2:1 to 5:1. The high-density (1.46) fraction appeared homogeneous by exclusion chromatography and recentrifugation in CsBr and had an amino acid composition characteristic of a mucin-type structure (threonine, serine, proline, glycine, and alanine comprise two-thirds of the amino acid residues). The carbohydrate, which is nearly 80% by weight, was O-glycosidically linked via GalNAc, sulfated (5.4% by weight), and contained fucose, galactose, glucosamine, galactosamine, and sialic acid. The low-density fraction had an amino acid composition distinct from that of the high-density fraction (threonine, serine, proline, glycine, and alanine comprise 51% of the amino acid residues) and a lower sulfate content. The size distribution of the saccharides in the low-density fraction was similar to that of the high-density fraction; the same sugars were present although the ratios were different. The low-density fraction contained 3 times more noncovalently associated lipid than did the high-density fraction. Several distinct classes of lipids were identified. Neutral lipids (mono-, di-, and triglycerides, cholesterol, and cholesteryl esters) comprised 56% by weight of the total lipid. Glycolipids and phospholipids were also identified. Palmitate (16:0), stearate (18:0), and oleate (18:1) were the major fatty acids in all classes of lipids.

Human tracheobronchial secretions consist of a heterogeneous population of macromolecules, salts, and water. Respiratory mucus, as well as mucus from other sources (e.g., gastric, salivary, and cervical mucus), serves as a lubricative and protective barrier and, in addition, provides a clearance mechanism for particulate matter from the respiratory tract.

Human tracheobronchial mucus has been characterized in terms of total protein, carbohydrate, lipid, and inorganic salts (Basch et al., 1941; Masson et al., 1965; Chernick & Barbero, 1959). Rheological properties have also been studied (Mitchell-Heggs, 1977; Charman & Reid, 1972, 1973).

The major glycoprotein present in tracheobronchial secretions is a large, carbohydrate-rich glycoprotein whose macromolecular structure regulates the viscoelastic properties of mucus. Isolation and characterization of the individual components from mucus are required to understand structure–function relationships.

Mucus contains several components in addition to the major mucin glycoprotein, most of which are lower molecular weight proteins and serum-type glycoproteins (Havez et al., 1968; Roussel et al., 1975; Boat et al., 1976; Feldhoff et al., 1976, 1979; Rose et al., 1979). Resolution of these components often involves the use of exclusion chromatography (Boat et al.,

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1976; Feldhoff et al., 1979; Rose et al., 1979) or ion-exchange chromatography (Lamblin et al., 1977; Roussel et al., 1975) to separate the high molecular weight, polyanionic mucin glycoprotein from lower molecular weight serum components. Another separation technique which has proved quite valuable is density gradient centrifugation in CsBr/CsCl solutions. This technique has been used preparatively for bronchial mucin glycoproteins (Creeth et al., 1977) as well as for blood group substances (Creeth & Denborough, 1970), pig gastric and intestinal mucus (Starkey et al., 1974; Mantle & Allen, 1981), and human gastric mucus (Pearson et al., 1980). Studies on these types of secretions have shown the presence of tightly, but noncovalently bound, protein and have provided detailed information on the macromolecular organization of mucus components.

Detailed studies on "normal" human tracheobronchial secretions, however, are often incomplete because of difficulties in collecting adequate amounts of secretions for chemical characterization. Previous studies from our laboratory have accordingly reported on mucin glycoproteins isolated from the tracheobronchial secretions of an asthmatic patient (Feldhoff et al., 1979). This paper describes the isolation, purification, and characterization of mucin glycoprotein from bronchial secretions of patients who have undergone surgery for nonpulmonary illness and from primary explant organ cultures of human tracheal epithelium.

## Materials and Methods

*Collection of Bronchial Aspirate Material.* Mucus was collected every 8 h by sterile aspiration at room temperature from seven different patients who had undergone surgery for nonpulmonary illness. Blood group status of these patients was determined by hemagglutination inhibition using anti-A and anti-B serum and a 2% suspension of red blood cells (Morgan & Watkins, 1951). Three patients exhibited blood group A activity, one patient blood group B activity, and three patients no detectable blood group A or B activity. Samples were stored in the freezer if not used immediately. Samples used immediately were diluted 1:1 with phosphate-buffered saline (PBS)[1] containing 0.02% $NaN_3$ and stirred at 4 °C for 24 h and then centrifuged at 10000 rpm (12000*g*) for 20 min to remove a small amount of cellular debris. No visible gellike material was present in the pellet. This pellet contained less than 10% by weight of the starting material and was not examined further. The resulting supernatant which contained 90–95% of the carbohydrate content of the original material was dialyzed against distilled $H_2O$ at 4 °C for 48 h and then lyophilized.

*Primary Explant Culture of Tracheal Epithelium.* Segments of trachea were obtained from one subject at autopsy within 4 h after death and were cultured on the basis of the procedure of Boat et al. (1977). Epithelial cells were removed from the segments by gentle scraping with a scalpel and tweezers and then transferred to Gibco medium 199 + glutamine (685 $\mu$M) in culture plates (2–3 mL of medium/plate). [$^3$H]Glucosamine (100 $\mu$Ci; New England Nuclear, specific activity = 20.2 Ci/mmol) and [$^{14}$C]leucine (10 $\mu$Ci; Amersham, specific activity = 35 mCi/mmol) were added to each plate. The plates were incubated at 37 °C in a 5% $CO_2$/95% air atmosphere, and medium (containing isotope) was changed every 24 h. The epithelial cells were cultured for 3–4 days. Pooled, spent medium was dialyzed against $H_2O$ at 4 °C for 3 days and then lyophilized.

*Analytical Methods.* Chromatographic column effluents and CsBr density gradient fractions were screened for neutral sugar by the phenol/$H_2SO_4$ method (Dubois et al., 1956) with galactose as a standard and for protein by measuring the absorbance at 280 nm. Specific sugar components were identified by gas–liquid chromatography of their alditol acetates after hydrolysis in either 2 N HCl or 2 N trifluoroacetic acid (Sawardekar et al., 1965; Lehnhardt & Winzler, 1968) or by methanolysis of sugars in 1.4 N methanolic HCl at 85 °C for 24 h, followed by conversion to the trimethylsilyl derivatives prior to chromatography (Clamp et al., 1971). Glucosamine and galactosamine were also determined on the amino acid analyzer after hydrolysis for 8 h at 100 °C in 4 N HCl.

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Samples for amino acid analysis were hydrolyzed in sealed ampules for 24 h at 110 °C with 6 N HCl. Analyses were performed on a Beckman 120C or 121 amino acid analyzer or on a Dionex amino acid/peptide analyzer MBFSS kit.

Sulfate was determined by the method of Spencer (1960) after hydrolysis for 12 h at 110 °C in 6 N HCl. Uronic acid was determined by a modified carbazole method (Davidson, 1966).

*Ouchterlony Immunodiffusion of Sepharose CL-4B Included Fractions.* Ouchterlony immunodiffusion was performed according to the procedure of Williams (Williams & Chase, 1971). Two samples of unfractionated bronchial mucus (samples C.L. and K.W.) and one sample of fractionated lower molecular weight components (C.L.) in 0.15 M phosphate buffer, pH 7.2, were placed in the immunodiffusion wells, covered, and incubated in a humid atmosphere at room temperature for 24 h. Gels were stained with Buffalo Black for 2–6 h and destained with 2% acetic acid.

The antisera used were from commercial sources. Rabbit anti-whole human serum, rabbit anti-$\alpha_1$-acid glycoprotein (lot 78M), and goat anti-human serum albumin (lot G389) were from Miles Biochemicals. Goat anti-IgG (lot 231-78-13), goat anti-IgA (lot 231-78-21), and goat anti-whole human serum (lot 231-74-7) were from Bionetics.

*Electrophoretic Studies.* $NaDodSO_4$–polyacrylamide gel electrophoresis was performed in 5% polyacrylamide gels according to the method of Weber & Osborn (1969). Mucin (100–200 $\mu$g) was incubated in $NaDodSO_4$/$\beta$ME at 110 °C for 3 min and then applied to the gels. After electrophoresis, gels were stained for protein with Coomassie Brilliant Blue and for carbohydrate by the periodate–Schiff method (Fairbanks et al., 1971).

*CsBr Density Gradient Centrifugation.* Sepharose CL-4B void volume material from fractionation of crude bronchial aspirates or spent culture medium was subjected to CsBr density gradient centrifugation on the basis of the procedure of Creeth et al. (1977). Void volume material was suspended in phosphate buffer (16.7 mM $Na_2HPO_4 \cdot 7H_2O$, 16.7 mM $NaH_2PO_4 \cdot H_2O$, 33 mM NaCl, and 0.02% $NaN_3$) containing 42% (w/w) CsBr at a loading density of 1.43 g/cm$^3$ and stirred for 24 h at 4 °C. After the mixture was stirred, it was precentrifuged at 5000 rpm (3000*g*) for 5 min at 4 °C, and then the supernatant was transferred to 1 in. × 3.5 in. cellulose nitrate centrifuge tubes. After this centrifugation, 97% of the total neutral sugar remained in the supernatant. Tubes were filled to the top and capped. The samples were centrifuged for 67 or 85 h at 14 °C and 42000 rpm in a Beckman 60Ti

[1] Abbreviations: PBS, phosphate-buffered saline; MeOH, methanol; DPC, dipalmitoylphosphatidylcholine; $NaDodSO_4$, sodium dodecyl sulfate; $\beta$ME, $\beta$-mercaptoethanol; HOAc, glacial acetic acid; TLC, thin-layer chromatography.

rotor. After centrifugation, tubes were punctured and 1-mL fractions collected and assayed (after diluting 1:10 with $H_2O$) for protein and for neutral sugar. Fractions from centrifugation of labeled materials were counted in ACS II (Amersham). Densities were determined by weighing a 100-$\mu$L aliquot of each fraction.

In some cases, centrifugation was performed in 0.625 in. × 2.5 in. tubes in a Beckman type 50 rotor. All other conditions were identical with those described above except that 0.7–0.8-mL fractions were collected.

*Alkaline Borohydride Treatment.* High- and low-density fractions were treated with 1 M $NaBH_4$ in 0.1 N NaOH according to the procedure of Carlson (1966). A 0.1% solution of mucin in 1 M $NaBH_4$ in 0.1 N NaOH was incubated for 72 h at 37 °C in the dark under $N_2$. Following incubation, mixtures were neutralized with 4 N HOAc and evaporated to dryness by rotary evaporation. The residue was washed repeatedly (5 times) with 1000:1 MeOH:concentrated HCl, evaporating to dryness by rotary evaporation after each washing. The residue was then analyzed by chromatography on a Bio-Gel P-10 column (column dimensions 1 × 30 cm) in 0.1 N pyridinium acetate, pH 5.1.

*Lipid Extraction.* Mucin samples (50 mg) were extracted sequentially for 12 h at 4 °C in 30-mL Corex test tubes with 20 mL of 2:1 $CHCl_3$:MeOH, 10 mL of 2:1 $CHCl_3$:MeOH, and finally 5 mL of 1:2 $CHCl_3$:MeOH, based on a procedure discussed by Esselman et al. (1972). After each extraction, samples were centrifuged at 10000 rpm (12000*g*) for 20 min, and the supernatants were removed with a Pasteur pipet. Addition of a small volume of methanol (usually 5 mL) aided in pelleting the insoluble glycoprotein. The combined supernatants were filtered through coarse-grade filter paper and evaporated to dryness by rotary evaporation at 25 °C. Lipid residues were dried for 24 h in vacuo over $P_2O_5$ and then weighed. The delipidated mucins were suspended in $H_2O$ and then lyophilized.

*Silicic Acid Column Chromatography.* Lipid extracts were chromatographed on a Bio-Sil A (200–400 mesh) silicic acid column (Esselman et al., 1972). Heat-activated silicic acid (100 °C, 8 h) was equilibrated in $CHCl_3$, and then a 4-mL (1 × 5 cm) column was poured. The column was washed sequentially with 10 column volumes of $CHCl_3$, 10 column volumes of 9:1 acetone:MeOH, 10 column volumes of MeOH, and finally 10 column volumes of $CHCl_3$. Lipid residue, up to 15 mg suspended in 1 mL of $CHCl_3$, was applied to the column, and then the column was eluted sequentially with 5 bed volumes each of $CHCl_3$, 9:1 acetone:MeOH, and MeOH. Effluents from each different wash were collected separately and evaporated to dryness by rotary evaporation. The residues were resuspended in a small volume of elution solvent, transferred to weighed tubes, evaporated to dryness with $N_2$, dried in vacuo over $P_2O_5$, and then weighed.

The $CHCl_3$ fraction, containing neutral lipid, was further chromatographed on a 4-mL (1 × 5 cm) Bio-Sil A column (200–400 mesh) equilibrated in 6% benzene in hexane (Lo-Chang & Sweeley, 1963). Neutral lipid in 1 mL of 6% benzene in hexane was applied to the column, and the column was eluted sequentially with 5 column volumes each of 6% benzene in hexane, 20% benzene in hexane, 65% benzene in hexane, and $CHCl_3$. Effluents were collected and evaporated to dryness by rotary evaporation. Residues were resuspended in a small volume of elution solvent, transferred to weighed tubes, evaporated to dryness with $N_2$, dried in vacuo over $P_2O_5$, and then weighed. Lipids in these fractions were identified by TLC. Lipids in the acetone/MeOH fraction (glycolipid enriched) and the MeOH fraction (phospholipid enriched) were also identified by TLC, without further purification.

*TLC of Lipid Fractions.* Whatman silica gel K5 plates (250-$\mu$m thickness) used for lipid separation were heated at 100 °C for 12 h and used immediately upon being cooled to room temperature. Solvent systems used (all solvents were redistilled before use) were 90:10:1 hexane:diethyl ether:glacial acetic acid for separation of neutral lipids and 65:25:4 $CHCl_3$:MeOH:$H_2O$ or 65:35:8 $CHCl_3$MeOH:2.5% aqueous ammonia for separation of phospholipids and glycolipids. All fractions were dissolved in 2:1 $CHCl_3$:MeOH before being applied to the plates.

All lipids were visualized by 50% sulfuric acid charring or with $I_2$ vapors followed by spraying with a 1% starch solution. Glycolipids were specifically visualized with 2% $\alpha$-naphthol in ethanol followed by $H_2SO_4$ charring. Phospholipids were visualized with 2.5% ammonium molybdate in 2 N $H_2SO_4$ followed by 0.25% ascorbic acid. Phospholipids were also visualized with Phospray (Supelco). Choline-containing lipids were visualized with Chargaff's reagent (Stahl, 1965).

*Fatty Acid Analysis.* Fatty acids were determined as their methyl esters after treatment with 14% w/v $BF_3$/MeOH for 3 h at 110 °C. Methyl esters were separated and identified by gas–liquid chromatography on a column of 10% DEGS-PS 80/100 Supelcoport. The chromatography was conducted isothermally at 200 °C at a flow rate of 30 mL/min with $N_2$ as a carrier gas. The column was attached to a Perkin-Elmer 900 gas chromatograph equipped with a flame ionization detector.

*Equilibrium Sedimentation Analysis.* High- and low-density delipidated glycoproteins were examined by analytical equilibrium ultracentrifugation by using the meniscus depletion method (Yphantis, 1964). Prior to centrifugation, the sample in PBS was dialyzed against PBS for 24 h.

## Results

Results of all the analyses correspond to the mean value of the seven different samples. After centrifugation of crude bronchial aspirate material at 12000*g*, 90–95% of the total neutral sugar remained in the supernatant. Chromatography of the supernatant on Sepharose CL-4B (Figure 1) separated the material into an excluded fraction (peak I) containing a mucin-type glycoprotein and a heterogeneous included fraction (peak II) consisting of serum proteins and glycoproteins. The excluded fraction accounted for 65–75% of the total neutral sugar. Blood group activity in the crude aspirate material, measured by hemagglutination inhibition, was recovered in the excluded fraction. Amino acid analysis of this fraction (Table I) indicated a composition characteristic of a mucin-type structure: threonine, serine, proline, glycine, and alanine comprised 54% of the total amino acid residues. Aromatic amino acids (phenylalanine and tyrosine) and cysteine were low. Fucose and galactose comprised 30–40% by weight of the mucin glycoprotein. The hexosamines were present in equimolar ratios and accounted for 25–30% by weight of the mucin glycoprotein. Fraction II had an amino acid composition characteristic of serum-type components. The presence of mannose and extensive cross-reactivity with serum components after double immunodiffusion identified components in this fraction as circulating serum proteins and glycoproteins (Figure 2).

A similar type of elution profile was observed when labeled, spent medium from organ culture was chromatographed (Figure 3). A high molecular weight, excluded fraction (80% of the total [$^3$H]glucosamine) and a lower molecular weight included fraction (30% of the total [$^{14}$C]leucine) were observed.

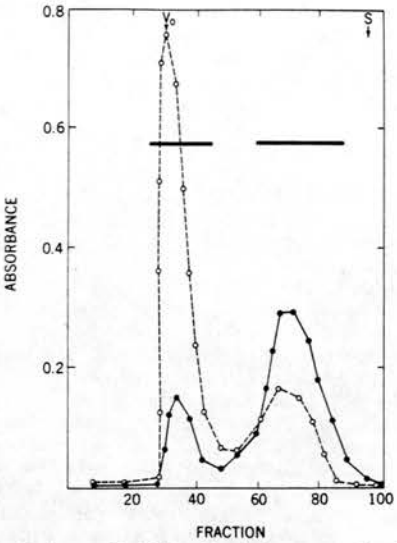


FIGURE 1: Sepharose CL-4B (5 × 95 cm) chromatography of crude bronchial aspirate material in 50 mM Tris, pH 8, containing 0.02% $NaN_3$. A 10 mg/mL solution of mucin in buffer was stirred at room temperature for 3 h and then centrifuged at 12000g for 10 min. The supernatant was applied to the column equilibrated in the buffer. 100 × 20 mL fractions were collected and screened for protein (●) and neutral sugar (○).

Table I: Amino Acid Composition of Bronchial Secretions after Chromatography on Sepharose CL-4B

| amino acid | residues/1000 residues peak I | peak II |
|---|---|---|
| lysine | 29 | 64 |
| histidine | 21 | 22 |
| arginine | 40 | 40 |
| cysteic acid | 4 | 1 |
| aspartic acid | 70 | 115 |
| threonine | 170 | 59 |
| serine | 110 | 59 |
| glutamic acid | 78 | 138 |
| proline | 103 | 79 |
| glycine | 72 | 63 |
| alanine | 86 | 75 |
| half-cystine | 15 | 35 |
| valine | 69 | 66 |
| methionine | 10 | 11 |
| isoleucine | 29 | 22 |
| leucine | 63 | 85 |
| tyrosine | 11 | 27 |
| phenylalanine | 21 | 41 |
| glucosamine[a] | 13 | 6 |
| galactosamine[a] | 12 | 1.5 |

[a] Expressed as percent by weight of the total glycoprotein.

$NaDodSO_4$–polyacrylamide gel electrophoresis of the excluded fractions in 5% polyacrylamide gels indicated the presence of lower molecular weight contaminants in both the bronchial aspirate and the organ culture materials. Rechromatography of the excluded fraction of Sepharose CL-4B also indicated the presence of contaminating protein, although greater than 95% of the total neutral sugar in the bronchial aspirate material remained in the excluded fraction. CsBr density gradient centrifugation was used to separate this contaminating protein from the mucin glycoprotein. A high-density (1.46) and a low-density (1.34) fraction were obtained from both bronchial aspirate material and tracheal organ culture (Figure 4A,B).

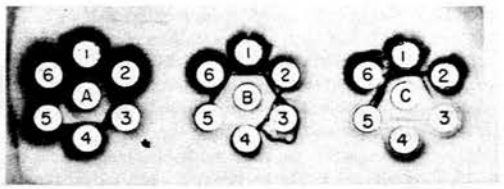


FIGURE 2: Ouchterlony immunodiffusion reactions of human tracheobronchial components (center walls) against antisera (outer wells). Center wells: A, unfractionated bronchial secretion (K.W.); B, low molecular weight fraction from Sepharose CL-4B chromatography; C, unfractionated bronchial secretion (C.L.). Outer wells: 1, rabbit anti-whole human serum; 2, anti-$\alpha_1$-acid glycoprotein; 3, anti-IgG; 4, anti-IgA; 5, blank; 6A, goat anti-whole human serum; 6B, anti-human serum albumin.

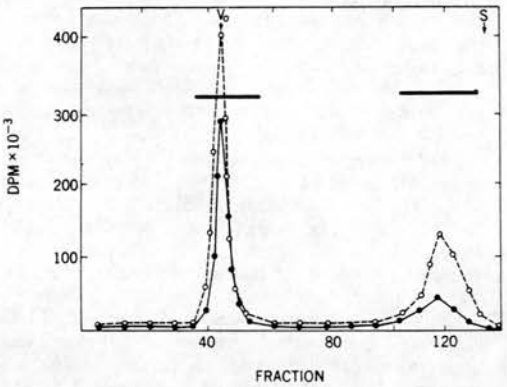


FIGURE 3: Sepharose CL-4B (2 × 65 cm) chromatography of labeled spent culture medium in 50 mM Tris, pH B, containing 0.02% $NaN_3$. 140 × 1.5 mL fractions were collected and aliquots of each fraction counted in ACS II with a liquid scintillation spectrometer. (○) [$^3$H]Glucosamine; (●) [$^{14}$C]leucine.

Table II: Amino Acid Composition of High- and Low-Density Fractions from Bronchial Aspirates

| amino acid | residues/1000 residues high density | low density |
|---|---|---|
| lysine | 26 | 45 |
| histidine | 26 | 27 |
| arginine | 35 | 43 |
| aspartic acid | 46 | 72 |
| threonine | 210 | 112 |
| serine | 127 | 110 |
| glutamic acid | 63 | 82 |
| proline | 120 | 87 |
| alanine | 96 | 96 |
| glycine | 91 | 104 |
| valine | 44 | 55 |
| methionine | 3 | 10 |
| isoleucine | 33 | 34 |
| leucine | 52 | 63 |
| tyrosine | 10 | 28 |
| phenylalanine | 10 | 33 |
| threonine + serine + proline + glycine + alanine | 644 | 509 |
| glucosamine[a] | 14 | 13 |
| galactosamine[a] | 11 | 4 |

[a] Expressed as percent by weight of the total glycoprotein.

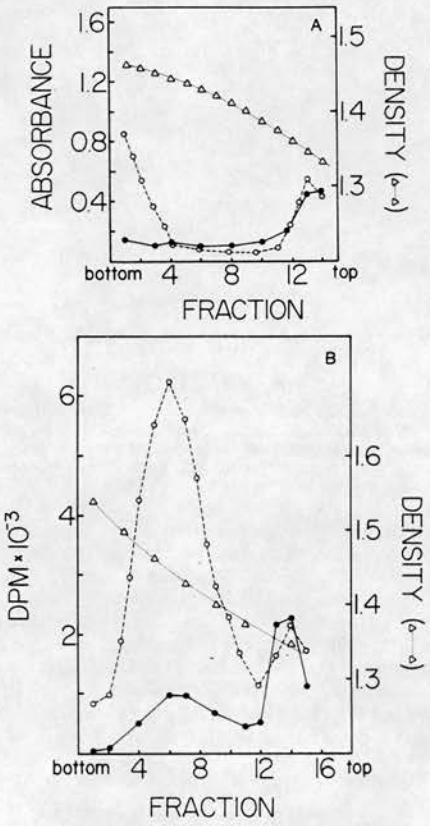


FIGURE 4: CsBr density gradient centrifugation of void volume material. (A) Bronchial aspirate material excluded on Sepharose CL-4B was loaded at a concentration of 1–2 mg/mL and centrifuged for 85 h at 42000 rpm. Starting density was 1.43 mg/mL. Fractions (0.7–0.8 mL) were collected and analyzed for protein (●) and neutral sugar (○). (B) Tracheal organ culture material excluded on Sepharose CL-4B was centrifuged as above but for 67 h. Fractions (0.7–0.8 mL) were collected and aliquots screened for [$^3$H]glucosamine (○) and [$^{14}$C]leucine (●).

Both high- and low-density fractions from bronchial aspirate material contained 30–40% neutral sugar and 10–20% protein by weight. The high-density fraction had an amino acid composition characteristic of a mucin-type structure (Table II): threonine, serine, proline, glycine, and alanine totaled 64% of the amino acid residues; aromatic amino acids were low (phenylalanine and tyrosine totaled 2% of the amino acid residues). The low-density fraction had an amino acid composition distinct from that of the high-density fraction: threonine, serine, proline, glycine, and alanine totaled 51% of the amino acid residues; aromatic amino acids (phenylalanine and tyrosine) were greater (6% of the total amino acid residues) than in the high-density fraction. The high- and low-density fractions from bronchial aspirate material had similar carbohydrate compositions, but the ratios of monosaccharides in the two fractions were different (Table III). The neutral sugar:hexosamine ratio was 2:1 in the high-density fraction and 3:1 in the low-density fraction. Sulfate was present in both the high-density (5.4% by weight) and low-density (3.6% by weight) fractions. Glucose was also detected in both the high- and low-density fractions. Delipidation removed most of the

Table III: Carbohydrate Analysis[a] of High- and Low-Density Fractions from Bronchial Aspirates

| sugar | % by weight of total glycoprotein: high density | % by weight of total glycoprotein: low density |
|---|---|---|
| fucose | 19 | 21 |
| galactose | 20 | 23 |
| *N*-acetylglucosamine | 14 | 13 |
| *N*-acetylgalactosamine | 11 | 4 |
| sialic acid (as NeuNAc) | 4 | 3 |
| glucose | 1 | 2 |
| sulfate | 5.4 | 3.6 |

[a] These analyses are on nondelipidated samples. Glucose is decreased in delipidated samples (less than 1% by weight of total glycoprotein in both the high- and low-density fractions).

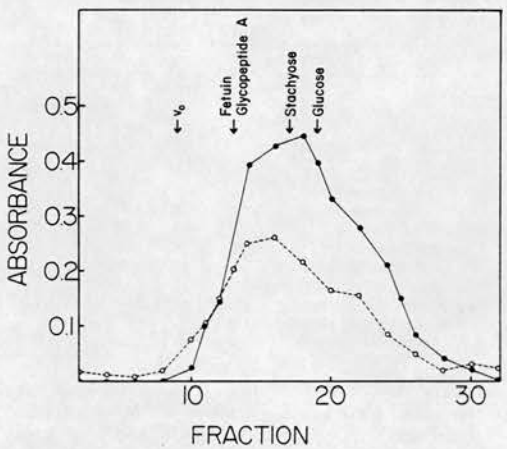


FIGURE 5: Bio-Gel P-10 (1 × 30 cm) chromatography in 0.1 N pyridinium acetate, pH 5.1, of high- (●) and low-density (○) fractions from bronchial aspirates after treatment with alkaline sodium borohydride. Aliquots of each fraction were screened for neutral sugar.

glucose, suggesting the presence of glycolipid. The remaining glucose may be a result of contamination from the dialysis tubing used during purification. Only trace amounts of mannose (<0.5% of the total carbohydrate) were detected in the high-density fraction. A slight amount of mannose was detected in the low-density fraction (1% of the total carbohydrate). Neither xylose nor uronic acid was detected in either the high- or the low-density fractions. The carbohydrate in both the high- and low-density fractions is linked to protein via Ser/Thr on the basis of susceptibility to alkaline sodium borohydride (Figure 5). The oligosaccharides of the high- and low-density fractions were similar in size distribution and ranged from 2–14 sugars.

Neither the high- nor the low-density fractions from tracheal organ culture were susceptible to chondroitinase ABC digestion as judged by their elution profile on Sepharose CL-4B. These data indicate the absence of proteoglycan in these fractions.

Equilibrium sedimentation analysis of the high- and low-density *delipidated* fractions (Figure 6) yielded molecular weights of $2.3 \times 10^6$ for the high-density fraction and $1.5 \times 10^6$ for the low-density fraction. The curvature of the plots showed a significant degree of polydispersity and/or aggregation with molecular weights ranging to $4.6 \times 10^6$ for the high-density component and to $5.6 \times 10^6$ for the low-density component.

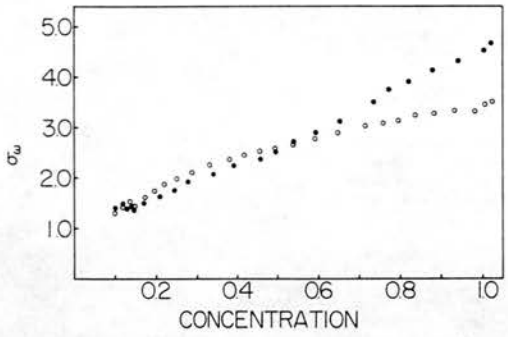


FIGURE 6: Equilibrium sedimentation analysis of the high- (○) and low-density (●) *delipidated* fractions from bronchial aspirate material excluded on Sepharose CL-4B.

Table IV: Lipid Composition of Mucin

| class of lipid | weight % of total lipid | % of total fatty acids as palmitate + stearate + oleate |
|---|---|---|
| neutral lipids | 56 | |
| monoglycerides | 5 | 61 for glycerides |
| diglycerides | 4 | |
| triglycerides | 20 | |
| cholesterol | 15 | |
| cholesteryl esters | 12 | 64 |
| glycolipid-enriched fraction | 26 | 63 |
| phospholipid-enriched fraction | 18 | 70 |
| phosphatidylcholine | | |
| other phospholipids | | |

The presence of noncovalently associated lipid was demonstrated by extraction of the high- and low-density fractions from bronchial aspirates with $CHCl_3$/MeOH. Three times as much lipid was associated with the low-density fraction than with the high-density fraction. This result suggested that variations in lipid binding gave rise to the different buoyant densities exhibited by the glycoproteins. As a test of this, (1) bronchial aspirate material excluded from Sepharose CL-4B was delipidated prior to density gradient centrifugation, and (2) the low-density fraction from bronchial aspirate material was delipidated and then rerun in density gradient centrifugation. The results of these two experiments are indicated in Figure 7. Both delipidation of bronchial aspirate material excluded from Sepharose CL-4B before centrifugation and delipidation of the low-density fraction, followed by recentrifugation, yielded only a high-density fraction. Analyses of material thus treated were substantially unchanged.

Lipid extraction and analysis were performed for seven different bronchial aspirate void volume fractions (Table IV), and the results are expressed as the mean value of the samples. The total weight percent of lipid associated with these fractions ranged from 5 to 15%. In all cases, neutral lipids (mono-, di-, and triglyercides, cholesterol, and cholesteryl esters) comprised the major class (56% of the total lipid). A glycolipid-enriched fraction (26% of the total lipid) and a phospholipid-enriched fraction (18% of the total lipid) were also identified. α-Naphthol-positive spots on TLC plates were designated glycolipids. Specific glycolipids were not identified, but those present had $R_f$ values similar to a commercially available (Sigma) preparation of crude cerebrosides. No gangliosides were detected, based on comparison of the mobilities of known gangliosides with the isolated lipids. In addition, no periodate–resorcinol-positive lipids were detected.

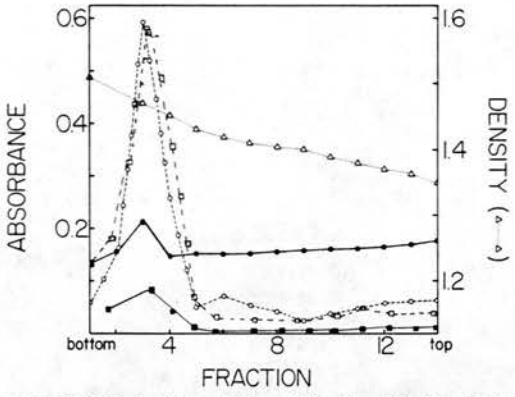


FIGURE 7: CsBr density gradient centrifugation of the low-density fraction after delipidation [(□) neutral sugar; (■) protein] and bronchial aspirate void volume material after delipidation [(○) neutral sugar; (●) protein]. Centrifugation conditions were identical with those described for Figure 4A.

Phosphatidylcholine was identified by comparison of the $R_f$ values of standard dipalmitoylphosphatidylcholine with those lipids present. Several lipids with $R_f$ values less than those for DPC were visualized but not identified.

The glycolipid fraction was estimated to be contaminated with 15–20% phospholipid based on spectrodensitometric scanning of the TLC plates. The phospholipid fraction was contaminated with 5–8% glycolipids. Palmitate ($C_{16:0}$), stearate ($C_{18:0}$), and oleate ($C_{18:1}$) were identified as the major fatty acids in all classes of lipids.

## Discussion

Mucin from bronchial aspirate material was chemically characterized after solubilization in aqueous buffer without the use of detergents or prior reduction. A similar result was reported from this laboratory in a study on mucus from an asthmatic patient (Feldhoff et al., 1979). This mild procedure allowed the isolation of mucin glycoprotein in a near-native state from seven different patients. Structurally similar macromolecules were isolated and characterized from these different secretions. Sepharose CL-4B chromatography separated mucin glycoprotein from lower molecular weight serum components, but entrapped contaminants were still present as judged by the presence of lower molecular weight components on $NaDodSO_4$–polyacrylamide gel electrophoresis.

CsBr density gradient centrifugation removed these contaminating components, and in addition, in four of the samples characterized, high-density and low-density mucin glycoproteins were identified. The high-density mucin glycoprotein appeared homogeneous by rechromatography on Sepharose CL-4B, by recentrifugation in CsBr, and by $NaDodSO_4$–polyacrylamide gel electrophoresis. In general, the isolated glycoprotein fraction met the following criteria: a single, albeit broad, peak on exclusion (A150M) or ion-exchange (DEAE-Sephadex A25) chromatography; no detectable amino-terminal end group; failure to enter a 0.5% agarose/2% acrylamide gel; no other protein component detectable by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (500 μg loading); absence of uronic acid (proteoglycan) and mannose (N-glycosidically linked glycoprotein); and complete susceptiblity of saccharides to alkaline borohydride catalyzed elimination. This component had an amino acid composition characteristic of a mucin-type structure similar to that reported

for bronchial glycoproteins obtained from a patient with cystic fibrosis (Rose et al., 1979) but a somewhat higher protein content than that reported from bronchial glycoproteins obtained from a patient with asthma (Feldhoff et al., 1979).

Carbohydrate analysis indicated the presence of fucose, galactose, glucosamine, galactosamine, and *N*-acetylneuraminic acid. The relative ratios of sugars were different than those reported in cystic fibrosis bronchial secretions (Rose et al., 1979; Roussel et al., 1975) and asthmatic bronchial secretions (Feldhoff et al., 1979). Sulfate was identified, probably esterified to galactose (Roussel et al., 1975). Mannose, xylose, and uronic acid, which are constituents of serum glycoproteins and proteoglycan, respectively, were not present. Equilibrium sedimentation analysis indicated the presence of aggregation and/or polydispersity of the glycoprotein whose molecular weight ranged from $2.3 \times 10^6$ to $4.6 \times 10^6$.

The low-density fraction present in some samples contained a mucin glycoprotein with a slightly altered composition from that of the high-density component, although the protein/carbohydrate ratios of the two components were the same (1:4). In addition, Sepharose CL-4B rechromatography indicated the presence of lower molecular weight components which were not further characterized. This low-density glycoprotein appeared homogeneous by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Previous studies using density gradient centrifugation have reported the presence of low buoyant density protein/glycoprotein components in blood group substances (Creeth & Denborough, 1970), bronchial glycoproteins from asthmatic bronchitic patients (Creeth et al., 1977), and glycoproteins from pig small intestinal mucus (Mantle & Allen, 1981; Mantle et al., 1981). These studies have not reported the presence of mucin-like glycoproteins of low buoyant density. The apparent low density appears to be a result of variations in lipid binding which can give rise to aggregates with different buoyant density. There is a slight alteration in carbohydrate and/or protein composition, but the overall mucin structure is present. These data suggest that in addition to the clustered, hydrophilic sugar domain a hydrophobic region is present which can bind lipid and may thus provide a mechanism for clearance of such components from the respiratory tract.

It is not known why some of the bronchial aspirate samples did not contain a low-density glycoprotein fraction. Lack of this component is probably not a result of a lack of lipid binding specificity since all seven aspirate samples qualitatively contained the same lipids. The slight quantitative differences in the samples (5–8% variations in any given class of lipid) may be sufficient to prevent resolution of a low-density fraction by the techniques employed. Quantitative differences of total associated lipid, however, do not seem to affect whether a low-density component appears upon centrifugation.

Lipids have been identified in pulmonary lavage of patients with alveolar proteinosis (Sahu et al., 1976), in secretions (Chernick & Barbero, 1959) and sputum (Matthews et al., 1963; Galabert et al., 1981) from cystic fibrosis patients, and from lung lavage of patients with asthma and cystic fibrosis (Sahu & Lynn, 1977). In these studies, phosphatidylcholine was identified as the major lipid. Neutral lipids such as mono-, di-, and triglycerides, cholesterol, and cholesteryl esters were identified, and some glycolipids were identified (Sahu & Lynn, 1977).

Lipids present in mucus obtained from an asthmatic patient were found to be different than those described in the above studies. Lewis (1971) found neutral lipids (mono-, di-, and triglycerides, free fatty acids, cholesterol, and cholesteryl esters) to be the major class (64.8% of total lipid weight); phospholipids were also identified.

The lipids associated with the mucin glycoprotein from normal bronchial secretions were similar to those described by Lewis. Neutral lipids comprised the major class of lipid (56% of the total lipid weight); phospholipids and glycolipids were present. It should be noted that these lipids were isolated from fractionated mucus (i.e., Sepharose CL-4B void volume material) whereas lipids described in the above studies were isolated from whole mucus. Lhermitte et al. (1977) reported the presence of lipid associated with mucin glycoproteins obtained from a patient with chronic bronchitis. They found that two fractions obtained from affinity chromatography had associated lipid identified as neutral lipids (steroids, free fatty acids, and mono-, di-, and triglycerides) and polar lipids (cardiolipids, cephalins, lecithins, and sphingomyelins).

It is clear from the lipid analysis that the contribution of lipid to the overall architecture of mucus secretions cannot be overlooked. The analysis does not indicate any specificity of a given lipid (or class of lipid) for interacting with mucin glycoprotein nor does it address the structural requirement of the glycoprotein for lipid binding. In vitro lipid binding experiments using intact mucin glycoproteins and mucin glycoprotein fragments may yield answers to these questions.

Experiments with primary explant culture of human tracheal epithelium suggest that these cells produce macromolecular components similar to those isolated from bronchial aspirate materials. The high- and low-density components incorporate glucosamine into glycoconjugates. In addition, the high-density component is susceptible to alkaline sodium borohydride treatment. Fractionation of the alkaline borohydride treated high-density component on Bio-Gel P-10 gave an elution profile similar to that obtained by fractionation of the oligosaccharides obtained from alkaline borohydride treated bronchial aspirate high-density mucin glycoprotein.

These preliminary data suggest that tracheal epithelial cells in culture produced macromolecules with physical properties similar to mucin glycoproteins from bronchial secretions.

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*Biochimica et Biophysica Acta*, 697 (1982) 344–352
Elsevier Biomedical Press

BBA 91082

# INFLUENCE OF GLYCOSAMINOGLYCANS ON ENDOGENOUS DNA SYNTHESIS IN ISOLATED NORMAL AND CANCER CELL NUCLEI

## DIFFERENTIAL EFFECT OF HEPARIN

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(Received October 29th, 1981)

*Key words: Glycosaminoglycan; DNA synthesis; Heparin effect; Tumor*

**The influence of exogenously-added glycosaminoglycans and glycoproteins on DNA synthesis in isolated nuclei, from normal and malignant tissues, was investigated. Heparin stimulated DNA synthesis in normal cell nuclei at concentrations (heparin/DNA (w/w)<0.9) which inhibited DNA synthesis in tumor cell nuclei. At higher concentrations (heparin/DNA (w/w)>0.9) heparin inhibited DNA synthesis in both normal and tumor cell nuclei. The chondroitin-4 and 6-sulfates, heparan sulfate, cartilage proteoglycan, *N*-desulfated heparin, and glycophorin caused inhibition of DNA synthesis at all concentrations tested and in all nuclei examined. Hyaluronic acid, dermatan sulfate, keratan sulfate, $\alpha_1$-acid glycoprotein and fetuin had no significant influence on DNA synthesis in isolated nuclei.**

### Introduction

Following the report from this laboratory [1] of the detection of glycosaminoglycans in the nuclei of B16 mouse melanoma cells, the presence of these compounds in the nuclei of a variety of cultured animal cells and tissues has been reported [2–7]. These findings point to the generality of the occurrence of glycosaminoglycans in animal cell nuclei (see review by Stoddart [8]). In addition to the presence of the strongly anionic glycosaminoglycans, the wide distribution of sialoglycoproteins in cell nuclei has also been amply documented [1–3,6,7,9,10]. A related observation of interest is the detection of a sulfated and phosphorylated galactan in the nuclei of the slime mold *Physarum polycephalum* [11,12]. Since in our initial experiments over 90% of the glycosaminoglycans were sedimentable after exhaustive treatment of nuclei with DNAase, it was suggested that the nuclear glycosaminoglycans might be primarily membrane associated [1]. However, subsequent studies by us [6,7] and by other investigators [2] indicate that a major portion of the nuclear glycosaminoglycans are localized in the chromatin. The failure to release glycosaminoglycans by DNAase digestion may be due to the inaccessibility of the enzyme to the chromatin and to the formation of insoluble complexes between the released histones and anionic glycosaminoglycans.

Several classes of acidic polymers have been shown to influence nucleic acid synthesis in isolated nuclei (see review by Coffey et al. [13]). Indirect evidence for the possible involvement of acidic polysaccharides in nuclear functions has been provided by a number of investigators [14–19]. The ability of polyanionic polysaccharides such as heparin [20,21], gum tragacanth [22] and dextran sulfate [23] to inhibit tumor cell growth in vitro and in vivo has also been observed. We,

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therefore, undertook an investigation to determine the influence of exogenously added glycosaminoglycans and glycoproteins on DNA synthesis in isolated nuclei from normal (rat liver, regenerating rat liver, rat kidney) and from cancer (hepatomas, melanoma, neuroblastoma), tissues. Our studies presented here, show that the highly anionic glycosaminoglycans have differential effects on DNA synthesis in normal and cancer tissues. A preliminary report has been presented [24].

## Materials and Methods

*Cells and tissues.* Morris hepatoma 7777 [25] (provided by Dr. John Taylor, Department of Microbiology, M.S. Hershey Medical Center) was maintained by bilateral implantation into the thigh muscle of male Buffalo rats (Simonsen Laboratories, Gilroy, CA). Normal livers and regenerating livers (17 h after partial hepatectomy) were obtained from animals approx. 12 weeks of age. B16 mouse melanoma cells were grown as solid tumors in C57BL/6J mice [26]. Mouse neuroblastoma tumors [27] grown in A/J mouse and Morris hepatoma 3924A [28] grown in ACI rats were kindly provided by Dr. C.-L. Schengrund and Dr. K. Rose, respectively.

*Isolation of nuclei.* Nuclei from perfused normal and regenerating rat livers and from rat kidneys were isolated using a modification [5] of the procedure described by Lynch et al. [29]. Rat liver nuclei prepared by this method has been shown to exhibit semiconservative DNA synthesis [30] compared to nuclei obtained by other methods [31,32]. Nuclei from solid tumors (hepatoma, melanoma and neuroblastoma) were similarly isolated except that in some experiments a different homogenization medium (25 mM citric acid/0.5 mM $CaCl_2$/0.5 mM $MgCl_2$) was used. Similar results were obtained in DNA synthesis assays using tumor nuclei isolated by the two methods. The absence of contaminating organelles in liver and hepatoma nuclei was established by assays for marker enzymes and by electron microscopic examination [1,5,7]. All preparations of nuclei were routinely monitored for purity by light microscopy.

*DNA synthesis in isolated nuclei.* Assay of DNA synthesis was done in triplicate in 0.25 M sucrose/50 mM Tris-HCl (pH 7.5)/25 mM KCl/7 mM $MgCl_2$/5 mM mercaptoethanol at 37°C using the following final concentrations of nucleoside triphosphate, 0.3 mM for dATP, dCTP and dGTP, 1 mM for ATP and 0.06 mM (5 $\mu$Ci) for [$^3$H]dTTP [17]. Nuclei (100–160 $\mu$g DNA/ml), cold nucleoside triphosphates and glycoconjugate in a final volume of 1 ml were mixed in siliconized glass test tubes, preincubated for 15 min, and the reaction started by addition of [$^3$H]dTTP. After incubating at 37°C for the appropriate time, the reaction was terminated by addition of cold 10% trichloroacetic acid/0.1 mM dTTP/10 mM sodium pyrophosphate. The radioactivity in the trichloroacetic acid supernatant was practically all (99%) dialysable, indicating that the labeled DNA was completely precipitated under the conditions used. The precipitate was washed three times with 5% trichloroacetic acid/10 mM sodium pyrophosphate. Control experiments showed that all unincorporated [$^3$H]dTTP was removed after three washes and that the use of siliconized tubes prevented the precipitate from sticking to the glass. The pellet was dissolved in 2% SDS (1 ml), mixed with 10 ml of ACS II scintillant (Amersham) and radioactivity determined by scintillation counting on an Intertechnique SL4000 spectrometer equipped with a DPM calculating module.

Controls in which the reaction mixtures were heated (100°C, 15 min) after preincubation and before the addition of [$^3$H]dTTP were included in every experiment. The radioactivity associated with the trichloroacetic acid precipitate in these controls was about 3% for normal and regenerating liver nuclei and about 0.3% for hepatoma nuclei compared to the radioactivity in trichloroacetic acid precipitates of the corresponding unheated assays. Further, the amount of radioactivity in the trichloroacetic acid precipitate of the heated controls was independent of incubation time. The influence of glycoconjugates on DNA synthesis for a 3 h time period are reported, unless otherwise stated or implied.

*Assay for protease and nuclease activities.* The glycosaminoglycan and glycoprotein samples were all tested for contaminating protease and nuclease activities under conditions identical to those used for the measurement of DNA synthesis. Azocasein

(Calbiochem, La Jolla, CA) and [$^{14}$C]methemoglobin (New England Nuclear, Boston, MA) were used as substrates for proteases. [$^{3}$H]DNA (isolated from regenerating livers of rats injected with [$^{3}$H]dTTP) and [$^{32}$P]tRNA from *Escherichia coli* (kindly provided by Dr. R. Coleman of this department) were used as substrates for nucleases. All samples were found to be free of these enzyme activities with the exception of a commercial sample of hyaluronic acid (Worthington) which had contaminating protease activity.

Desulfation of sulfated glycosaminoglycans was done according to the method of Nagasawa et al. [33]. The *N*-desulfated heparin was re-*N*-acetylated as described by Cifonelli [34]. The DNA content of nuclei was determined by the method of Burton [35] using calf thymus DNA as standard. Hexosamine was assayed after hydrolysis of the glycosaminoglycans (6 N HCl, 100°C, 12 h) by the method of Dische [36] or on an amino acid analyzer. The values were corrected for the decomposition of hexosamines during hydrolysis. Sulfate was determined by the barium chloranilate method [37]. Deoxynucleoside triphosphates were purchased from Sigma (St. Louis, MO) and adenosine triphosphate from Calbiochem (La Jolla, CA). [$^{3}$H]dTTP (70–80 Ci/mmol) was obtained from New England Nuclear (Boston, MA). The sources of the glycoconjugates used are given in the text. Cellulose acetate electrophoresis of glycosaminoglycans was carried out in 0.1 M pyridine/acetic acid, pH 5.0 at 1 mA/cm for 20 min. The glycosaminoglycans were detected by staining with Alcian blue (0.5% acetic acid) and their mobility relative to that of beef lung heparin ($^{®}$Hep) calculated.

## Results

The time course of DNA synthesis in the various nuclei is shown in Fig. 1; in all cases the reaction was linear during the initial hour. The reaction was also linear at DNA concentrations tested up to a level of 200 μg/ml (data not given). Incorporation of [$^{3}$H]dTTP into acid insoluble materials was lowest in normal quiescent liver nuclei and slightly higher in regenerating liver nuclei, as expected. In hepatoma nuclei, DNA synthesis was 5–8-fold higher than in normal liver

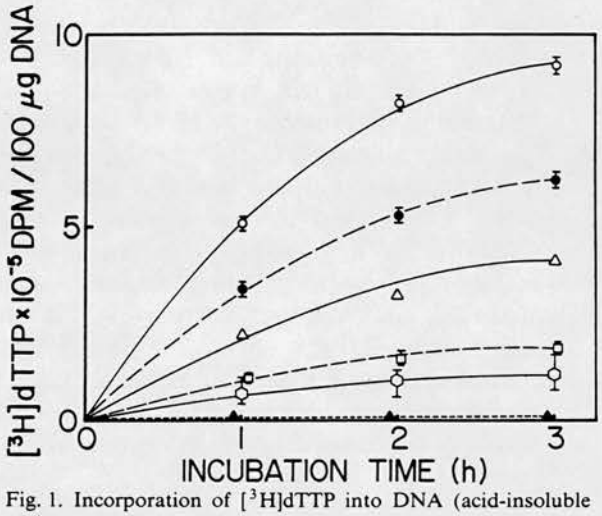


Fig. 1. Incorporation of [$^{3}$H]dTTP into DNA (acid-insoluble material) in isolated nuclei as a function of time. Nuclei from Morris hepatoma 7777 25 (○——○), 32 (●------●) or 47 (△——△) days after transplantation; nuclei from regenerating rat liver 17 h post-hepatectomy (□------□); nuclei from rat liver (○ ——— ○); heated (100°C, 15 min) rat liver nuclei (▲------▲).

nuclei. The highest rate of incorporation was found in hepatoma nuclei at early stages of growth (25 days after transplantation); this level of activity gradually decreased with increasing age of the tumor. This is apparently due to the increase in proportion of dormant and necrotic cells in the tumor. Routinely, hepatomas at 25–32 days of growth were used in our experiments.

In all cases, the incorporation of [$^{3}$H]dTTP into the nuclei was drastically reduced (95%) if unlabelled dTTP at 0.3 mM was included in the incubation mixture. DNA synthesis in the isolated nuclei was also inhibited 80–95% by 5–10 mM *N*-ethylmaleimide (Sigma, St. Louis, MO). This compound is reported to be a specific inhibitor of the DNA replicating enzyme, DNA polymerase α and is considered to have little or no effect on the DNA repair enzyme, DNA polymerase β [38]. It is thus likely that the major activity determined in our assay is that associated with replication. Further experiments are necessary to substantiate this observation.

*Effects of exogenously added hyaluronic acid (100 μg/100–160 μg DNA) on DNA synthesis*

Hyaluronic acid from various tissues and

sources (human umbilical cord: Boehringer Mannheim, F.R.G.; hog skin: Seikagaku Kogyo, Tokyo, Japan; bovine vitreous humor: Sigma, St. Louis, MO., Millipore, Freehold, NJ and isolated by K.F.) had no significant effect on DNA synthesis in isolated nuclei. Vitreous humor hyaluronic acid (from Worthington) caused inhibition, but this was proved to be due to a protease contaminant. Inclusion of soybean trypsin inhibitor in the assay mixture containing the Worthington hyaluronic acid restored DNA synthesis to within 10% of the control activity. Further, purification of this hyaluronic acid by chromatography on DEAE-Sepharose removed the contaminating component responsible for the inhibition.

*Effects of exogenously added sulfated glycosaminoglycans (100 μg/100–160 μg DNA) on DNA synthesis*

The results of a large number of experiments are summarized in Table I. Dermatan sulfate and keratan sulfate, each from two different sources did not have significant effects on DNA synthesis in any of the nuclei tested.

Among the other sulfated glycosaminoglycans tested, chondroitin-6-sulfate (three different samples) and cartilage proteoglycan inhibited DNA synthesis in hepatoma by 40–45% but caused lesser inhibition of DNA synthesis in normal and regenerating liver nuclei. After additional purification of the chondroitin-6-sulfate (Seikagaku Kogyo

TABLE I

EFFECT OF SULFATED GLYCOSAMINOGLYCANS ON ENDOGENOUS DNA SYNTHESIS IN ISOLATED NUCLEI FROM RAT LIVER, REGENERATING LIVER AND HEPATOMA

n.d. is not determined.

Several preparations of each of the sulfated glycosaminoglycans from different tissue sources and origins (commercial or gifts) were tested. The different preparations showed similar effects, however, quantitative differences were present. The data presented are representative. 100 μg sulfated glycosaminoglycan versus nuclei equivalent to 100–160 μg DNA is used in each experiments. $R_{Hep}$ electrophoretic mobility on cellulose acetate relative to bovine lung heparin. Kav is calculated from the elution profile on a Bio-Gel P-60 column using [$^3$H]porcine submaxillary gland mucin ($M_r$ 830000) and [$^{14}$C]glucose as markers for $V_0$ and $V_t$, respectively. Mean ± S.E.M., number of experiments is in parentheses.

| Glycosaminoglycan (Tissue source) | Sulfate / Hexosamine | $R_{Hep}$ | Kav ($\times 10^{-2}$) | [$^3$H]dTTP incorporated into DNA in 3 h; Percent control values | | |
|---|---|---|---|---|---|---|
| | | | | Normal liver | Regenerating liver | Morris hepatoma 7777 |
| Dermatan sulfate [a] (pig skin) | 1.11 | 0.84 | 6.45 | 88.5 ± 20 (3) | 89.7 | 84.9 ± 7.0 (2) |
| Keratan sulfate [b] (human costal cartilage) | 1.24 | 0.83 | 4.84 | 103.0 ± 1.0 (2) | 98.6 ± 7.4 (2) | 132 ± 10 (2) |
| Proteoglycan [c] (bovine nasal septum) | 0.72 | n.d. | n.d. | 91.6 ± 14 (4) | 83.3 ± 4.0 (3) | 59.6 ± 2.9 (2) |
| Chondroitin-4-Sulfate [b] (river sturgeon notochord) | 1.03 | 0.86 | 6.35 | 67.3 ± 4.1 (2) | n.d. | 79.0 ± 2.7 (2) |
| Chondroitin-6-Sulfate [d] (shark cartilage) | 1.25 | 0.92 | 3.23 | 76.4 ± 13 (7) | 69.3 ± 4.1 (3) | 55.1 ± 7.9 (3) |
| Heparan Sulfate [b] (bovine liver) | 1.17 | 0.86 | n.d. | 16.6 ± 3.3 (3) | n.d. | 30.4 |
| Heparin [b] (bovine lung) | 2.77 | 1.00 | 22.2 | 242 ± 22 (5) | 183 | 13.1 ± 7.9 (6) |
| Chondroitin Sulfate H [e] (hagfish notochord) | 1.65 | 0.93 | n.d. | 270 ± 13 (2) | n.d. | 16.1 ± 4.7 (3) |

[a] Purchased from Sigma.
[b] Kindly donated by Dr. M.B. Mathews, University of Chicago, Chicago, IL.
[c] Gift from Dr. E.A. Chandrasekaran, University of Georgia, Athens, GA.
[d] Obtained from Seikagaku Kogyo.
[e] Isolated by K.F.

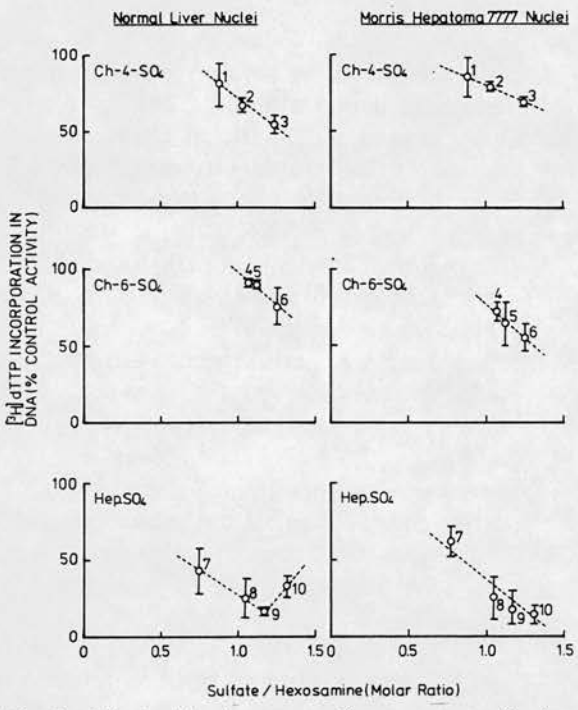


Fig. 2. Relationship between sulfate content of glycosaminoglycans and the degree of inhibition of DNA synthesis in nuclei isolated from rat normal liver (left panel) and Morris hepatoma 7777 (right panel). Glycosaminoglycans with different degree of sulfation were purchased from Sigma (1 and 5), Seikagaku Kogyo (3 and 6) or donated by Dr. M.B. Mathews (2, 4 and 9) and by Dr. A. Linker (7, 8 and 10), respectively.

Tokyo) and proteoglycan by DEAE-Sepharose column chromatography and CsCl density gradient centrifugation [39], respectively, the same results were obtained. Increasing concentrations of chondroitin-6-sulfate showed increased inhibition of DNA synthesis in normal liver nuclei. Desulfated chondroitin-6-sulfate, in which about 90% of the sulfate had been removed had no effect on DNA synthesis in any of the nuclei tested. Chondroitin-4-sulfates (three different samples) inhibited DNA synthesis to varying degrees in the different nuclei. Heparan sulfates (four different samples) showed marked inhibition of DNA synthesis in both liver and hepatoma nuclei. The degree of inhibition of DNA synthesis in nuclei by the chondroitin sulfates and heparan sulfate is correlated with the sulfate to hexosamine ratio (Fig. 2). The electrophoretic mobility ($^{R}$Hep) in pyridine/acetate buffer (pH 5.0) was also correlated to the degree of inhibition but the correlation was not as marked (data not given). However, additional studies are necessary to further substantiate these observations since the molecular size of these sulfated glycosaminoglycans and charge distribution may also be factors.

Heparin (two samples) and chondroitin sulfate H (polysulfated dermatan sulfate) showed contrasting effects on DNA synthesis in the different nuclei. Whereas, these compounds stimulated DNA synthesis in nuclei from normal tissues (rat liver and regenerating rat liver), at equivalent concentrations they consistently inhibited DNA synthesis in nuclei from the malignant (hepatoma) tissue (Table I).

The effects of heparin and its derivatives over a range of concentrations on DNA synthesis in normal liver and hepatoma nuclei are illustrated in Fig. 3. It was evident that in normal liver nuclei, low levels of heparin (heparin/DNA (w/w) $< 0.9$) stimulated DNA synthesis, the maximum stimulation was exhibited at 40 $\mu$g heparin/100 $\mu$g DNA. At higher levels of heparin (heparin/DNA (w/w) $> 0.9$) a marked inhibition is noted and at 130 $\mu$g heparin/100 $\mu$g DNA, the inhibition of DNA synthesis was essentially total (Fig. 3a). The effect of heparin on DNA synthesis in regenerating rat liver and rat kidney nuclei was similar to that observed in liver nuclei. With increasing heparin concentration for a fixed amount of nuclei isolated from regenerating rat liver or rat kidney, the

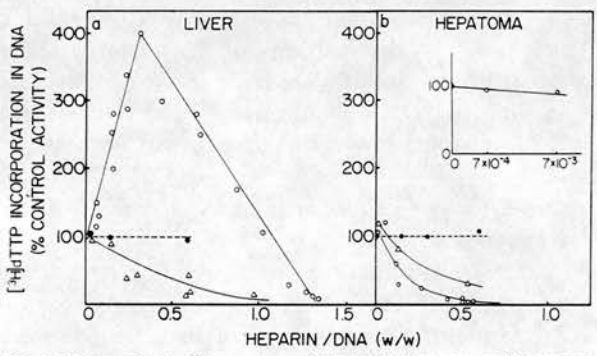


Fig. 3. Dose effect of heparin and its derivatives on endogenous DNA synthesis in nuclei isolated from (a) rat liver (b) Morris hepatoma 7777. Heparin (○——○); desulfated heparin (●- - - - -●); *N*-desulfated re-*N*-acetylated heparin (△——△). The effect of very low doses of heparin on DNA synthesis in hepatoma nuclei is shown in the inset.

stimulation of DNA synthesis was enhanced reaching a level in regenerating liver nuclei similar to that observed in normal liver nuclei at a weight ratio of heparin to DNA of 0.4; in kidney nuclei, a 3-fold enhancement compared to control level was observed at a heparin DNA ratio (w/w) of 0.4. At higher ratios, the degree of stimulation gradually decreases and finally a strong inhibition of DNA synthesis in both types of nuclei is noted. The observed effects of heparin on DNA synthesis in liver nuclei are in general agreement with the results of Kraemer and Coffey [14] utilizing exogenous *E. coli* DNA polymerase; Cook and Aikawa [17] using the endogenous DNA polymerase in isolated rat liver nuclei and Shimada and Terayama [40] using the endogenous DNA polymerase in isolated rat brain nuclei.

In contrast to the stimulation of DNA synthesis in normal liver nuclei at low concentrations and inhibition at higher concentrations, heparin showed no stimulation of DNA synthesis in hepatoma nuclei even at very low concentrations (see inset Fig. 3b). At concentrations of 10 μg heparin/100 μg DNA and higher, heparin consistently inhibited DNA synthesis in hepatoma nuclei. The inhibition was virtually complete at about 50 μg heparin/100 μg DNA. These data are in sharp contrast to the 16- and 26-fold stimulation by heparin of DNA synthesis in rat hepatoma (9618A and 5123C) nuclei reported by Coffey et al. [13]. However, their studies were done with exogenous DNA polymerase using [$^3$H]dTMP and a 30 min incubation and may not be directly comparable to ours. Desulfated heparin showed no influence on DNA synthesis in any of the nuclei tested. However, *N*-desulfated re-*N*-acetylated heparin inhibited DNA synthesis in both normal liver and hepatoma nuclei but to different degrees (Fig. 3).

*Effects of exogenously added glycoproteins on DNA synthesis*

In contrast to the striking effects shown by some of the sulfated glycosaminoglycans, glycoproteins tested showed either little or no effect on nuclear DNA synthesis. Glycophorin (100 μg/100–160 μg DNA) showed 47, 51 and 20% inhibition in nuclei from normal liver, regenerating liver and hepatoma, respectively. Fibronectin (47 μg/100–160 μg DNA) showed between 20 to 36% stimulation but the significance of this could not be established due to the limited quantities of this sample available. $\alpha_1$-acid glycoprotein and fetuin (Sigma) at concentration of 200 μg/100–160 μg DNA showed no significant effect. A strong inhibition of DNA synthesis in all nuclei by the fetuin sample from Gibco (Cat. No. 918S, Spiro method) was shown to be due to the presence of zinc. After dialysis against ethylenediamine tetraacetic acid or chromatography on a column of DEAE-Sepharose the inhibitory activity of this fetuin sample was lost. Atomic absorption spectrometric analysis showed the presence of 12.8 μg zinc in 1 mg of this fetuin preparation. Addition of 2.6 μg zinc to the reaction mixture caused the same degree of inhibition of DNA synthesis as that given by 200 μg of the Gibco fetuin.

*Alterations in pH and $Mg^{2+}$ concentration of reaction mixture by heparin*

The possibility that the effects of the exogenously added heparin on DNA synthesis were due to alterations in the pH and/or magnesium concentration of the reaction mixture was investigated. The change in pH of the complete assay

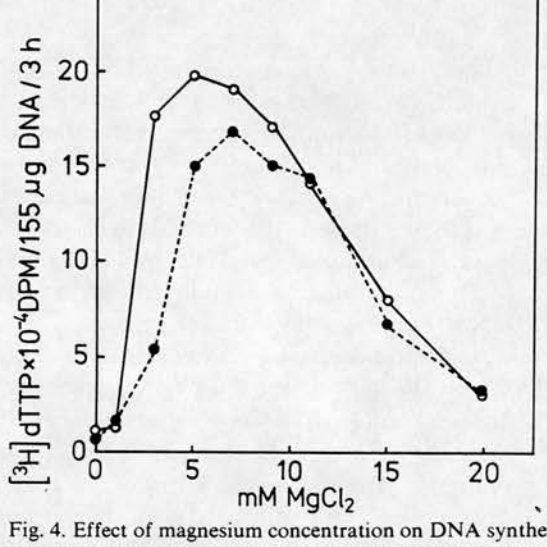


Fig. 4. Effect of magnesium concentration on DNA synthesis in isolated nuclei from normal rat liver. The reaction mixture is identical with the exception that the $MgCl_2$ concentration is varied from 0 to 20 mM in the absence (○——○) and presence (●------●) of 150 μg heparin/155 μg DNA.

mixtures with and without 200 μg heparin were identical. In the both cases the pH dropped from 7.5 to 7.40 and 7.35 after 1.5 and 3 h of incubation, respectively. The effect of $Mg^{2+}$ concentration on DNA synthesis in normal liver nuclei in the absence and presence of heparin is illustrated in Fig. 4. It is clear that the inhibition of DNA synthesis in liver nuclei (155 μg DNA) by heparin (150 μg) is not due to limiting $Mg^{2+}$ concentration. In fact, both in the absence and presence of heparin the optimal $Mg^{2+}$ concentration for DNA synthesis is between 5 to 7 mM. The concentration used in our routine incubation mixture was 7 mM.

*Dose effect of heparin on endogenous DNA synthesis in nuclei isolated from various tumors*

Experiments were done to determine whether the inhibition by heparin of DNA synthesis in Morris hepatoma 7777 nuclei was a phenomenon common to nuclei of malignant cells. Heparin inhibited DNA synthesis in nuclei from B16 mouse melanoma, mouse neuroblastoma and Morris hepatoma 3924A at both low and high concentrations. The degree of inhibition in melanoma and neuroblastoma was, however, lower than that observed with hepatoma nuclei at comparable concentrations of heparin. This may be partly due to the use of these tumor tissues from rather late stages of growth in comparison to the more actively growing hepatoma tissue.

## Discussion

Based on our results, the glycoconjugates may be classified into three groups depending on their influence on DNA synthesis. The first group, consisting of $\alpha_1$-acid glycoprotein, fetuin, hyaluronic acid, dermatan sulfate, keratan sulfate, desulfated chondroitin-4,6-sulfate and desulfated heparin, has no effect on DNA synthesis. The second group, consisting of the chondroitin sulfates, proteoglycan, heparan sulfate, *N*-desulfated heparin and glycophorin cause inhibition of DNA synthesis in both normal and cancer cell nuclei. The most interesting observation was the differential effect caused by the third group of glycosaminoglycans consisting of the highly sulfated heparin and chondroitin sulfate H.

It is evident from the results that the degree of anionicity does not by itself determine the nature of influence of glycoconjugates on DNA synthesis. Whereas proteoglycan and chondroitin-4-sulfate with sulfate to hexosamine ratios of 0.72 and 1.03 inhibits DNA synthesis, keratan sulfate and dermatan sulfate with higher degree of sulfation (sulfate to hexosamine ratios of 1.24 and 1.11) do not have any effect on DNA synthesis under identical conditions. However, within classes of glycosaminoglycans (for example, chondroitin sulfate and heparan sulfate) showing inhibition of DNA synthesis, the higher the degree of sulfation the higher the inhibition (Fig. 2). It is thus possible that dermatan and keratan sulfate samples of higher sulfate content than those tested in these studies may influence DNA synthesis.

Our results also show that differences in the molecular weight (size) among the glycosaminoglycans is not a sufficient factor to account for differences in their influence on DNA synthesis. Dermatan sulfate and chondroitin-4-sulfate have almost similar elution profiles on a Bio-Gel P-60 column (compare Kav, Table I) but the former has no effect and the latter inhibits DNA synthesis.

An important consideration in these studies is the ability of the various glycosaminoglycans to enter the nuclei. Preliminary results indicate that only a small portion of the added heparin enters the nuclei and only a fraction of this is associated with the chromatin. There was no significant difference in the ability of heparin to enter the normal or cancer cell nuclei. It is also found that saturation levels of heparin inside the nuclei is reached only after 2 to 3 h. Thus, in order to determine the maximum influence of exogenously added polyanions, it is necessary to carry out incubation for periods of about 3 h rather than the 30 min employed in previous studies [13,40].

The molecular mechanism of the influence of sulfated polysaccharides, in general, on DNA synthesis remains unknown. The fact that these acidic polysaccharides are capable of forming complexes with histones in vitro (unpublished data) and the finding that the effect on DNA synthesis by heparin was prevented by addition of histones [17] suggest that the interaction between the acidic polysaccharides and histones in the nuclei might be important. Further, the direct interaction of

glycosaminoglycans with DNA polymerases as observed in studies with purified bacterial polymerases [19] and animal polymerases (Refs. 41, 42 and unpublished data) may also be relevant.

It is difficult to explain the differential effects of the highly sulfated polysaccharides on DNA synthesis in normal and malignant cell nuclei. Differences in the structural organization of chromatin, endogenous glycosaminoglycan levels and structures (activities) of the polymerases may all be contributory factors. Thus, for example, it is possible that the heparin entering the normal cell nuclei initially interacts with chromatin increasing template availability thus resulting in the stimulation of DNA synthesis. At higher concentrations, the excess heparin could bind to DNA polymerase $\alpha$ [42] and inactivate it. In cancer cell nuclei the added heparin has no effect on template activity, either due to differences in chromatin organization or because the template activity is already maximal. As a consequence, even at low concentrations the added heparin interacts with the polymerase causing inhibition.

## Acknowledgements

We wish to thank Drs. M.B. Mathews, A. Linker and E.V. Chandrasekeran for their gifts of samples and Drs. J.M. Taylor, C.-L. Schengrund and K.M. Rose for providing the tumors used in this study. Our thanks are also due to Dr. E.A. Davidson for support and many valuable discussions throughout this study, and to him and Dr. S.T. Jacob for their critical reading of the manuscript. This work was supported in part by Grants Ca17686 and CA15483 from United States Public Health Service.

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*Biochimica et Biophysica Acta*, 719 (1982) 1–10
Elsevier Biomedical Press

BBA 21253



# STUDIES ON THE UNIQUE PRESENCE OF AN *N*-ACETYLGALACTOSAMINE RESIDUE IN THE CARBOHYDRATE MOIETIES OF HUMAN FOLLICLE-STIMULATING HORMONE

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(Received March 2nd, 1982)

*Key words: N-Acetylglucosamine; Carbohydrate structure; Follicle-stimulating hormone; (Human)*

**Glycopeptides located at asparagine residues at position 52 in the α-subunit and at position 7 in the β-subunit of human follicle-stimulating hormone (FSH) were obtained by pronase digestion of the intact human FSH molecule. The glycopeptide fractions were isolated by gel filtration and by high-voltage electrophoresis on paper at pH 6 and/or pH 3.5. Alkaline degradation studies performed on the α- and β-subunits of human follicle-stimulating hormone confirmed that carbohydrate moieties linked *O*-glycosidically to threonine or to serine are not present in either subunit. Carbohydrate composition of both α-52 and β-7 glycopeptides was similar. Sialic acid, mannose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and fucose were present in a ratio of 3:4:3:4:1:1 in both glycopeptides. The results of sequential enzymatic digestion revealed that the peripheral monosaccharide sequence of both the α-52 and β-7 glycopeptides consisted of three chains each containing the following sequence: sialic acid $\overset{\alpha}{-}$ galactose $\overset{\beta}{-}$ β-*N*-acetylglucosamine $\overset{\beta}{-}$ mannose. The composition of the undigested 'inner core' of both carbohydrate moieties was found to be similar and consisted of one residue/mol glycopeptide each of mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and fucose. The unique presence of GalNAc attached to an *N*-glycosidically linked GlcNAc was confirmed by hexosamine analysis on an amino acid analyzer, by thin-layer chromatography, by digestion with enzymes such as exo-α-*N*-acetylgalactosaminidase and endoglycosidases D and H, and by ion-exchange chromatography on DEAE-Sephacel. The monosaccharide sequence of the α-52 and the β-7 glycopeptides of human follicle-stimulating hormone is shown below and is identical to the one at the α-78 position.**

$$\left.\begin{array}{l}\text{NeuAc} \overset{\alpha}{-} \text{Gal} \overset{\beta}{-} \text{GlcNAc} \overset{\beta}{-} \text{Man} \\ \text{NeuAc} \overset{\alpha}{-} \text{Gal} \overset{\beta}{-} \text{GlcNAc} \overset{\beta}{-} \text{Man} \\ \text{NeuAc} \overset{\alpha}{-} \text{Gal} \overset{\beta}{-} \text{GlcNAc} \overset{\beta}{-} \text{Man}\end{array}\right\} \overset{\alpha}{-} \text{Man} - \text{GalNAc} - \text{GlcNAc} - \text{Asn}$$

Peripheral Region — Fucose, Inner core region

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Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; CG, chorionic gonadotropin; TSH, thyroid-stimulating hormone; NeuAc, *N*-acetylneuraminic acid; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Gal, galactose; Man, mannose; Asn, asparagine.

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## Introduction

Elucidation of the structure of the carbohydrate moieties of human FSH is important in understanding the metabolism, biological activity and structure-function relationship of the hormone. There are four asparagine-linked carbohydrate moieties in FSH; two are located at residues 52 and 78 in the $\alpha$-subunit sequence and the other two at residues 7 and 24 in the $\beta$-subunit sequence [1,2]. The monosaccharide sequence of the carbohydrate moiety at residue 78 of the $\alpha$-subunit has been reported [3]. The monosaccharide sequence was unique from other known glycopeptides in that an *N*-acetylgalactosamine residue was attached to the *N*-acetylglucosamine *N*-glycosidically linked in the 'inner core' (Fig. 1). Fetherstone et al. [4] and Kessler et al. [5] have reported an *N*-acetylglucosamine instead of an *N*-acetylgalactosamine in the inner core of the carbohydrate moieties of bovine luteinizing hormone and human chorionic gonadotropin. Since this report, *N*-acetylgalactosamine in an *N*-glycosidically linked oligosaccharide unit has been reported in ovine and bovine luteinizing hormones [6]; however, the galactosamine was found to be present in one of the peripheral chains rather than in the 'inner core'. Recently, Parsons and Pierce [7] have reported a sulfated terminal *N*-acetylgalactosamine in one of the peripheral chains of a carbohydrate moiety of bovine LH. These observations indicate

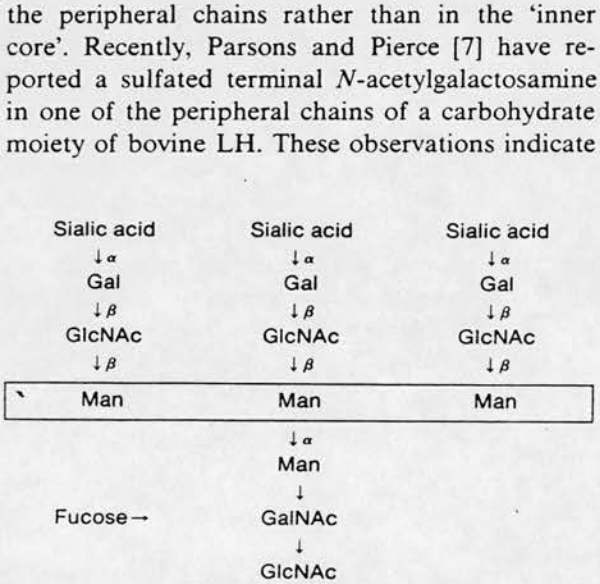



Fig. 1. Proposed common structure for the carbohydrate moieties of the human FSH-α-52 and human FSH-β-7 glycopeptides.

differences in the carbohydrate structures of closel related glycoprotein hormones such as bovine LH and human CG. In order to resolve the monosac charide sequence of carbohydrate moieties at posi tion 52 of human FSH-$\alpha$ and at position 7 o human FSH-$\beta$, further evidence was obtained b additional experiments to confirm the presenc and location of *N*-acetylgalactosamine in th carbohydrate moieties of human FSH.

## Materials and Methods

*Comparison of the N-acetylgalactosamine conten of human FSH, LH, thyroid-stimulating hormon (TSH), bovine LH, bovine TSH, and human CG* Human FSH, FSH-$\alpha$, FSH-$\beta$, LH, and TSH wer prepared in our laboratory from crude glycopro teins supplied to us by the National Pituitar Agency, MD. Bovine LH was supplied by th National Pituitary Agency, and human CG was gift from Drs. O.P. Bahl (SUNY, Buffalo, NY and R.E. Canfield (Columbia University, New York, NY). In addition, highly purified prepara tions of human FSH, FSH-$\alpha$, FSH-$\beta$, LH, LH-$\alpha$ LH-$\beta$, TSH and bovine TSH, supplied by th National Pituitary Agency, were also used in the comparison of galactosamine content of the hormones. Aliquots of 100 $\mu$g protein from each o the above hormones were hydrolyzed for 4 h a 105°C with 5.7 M HCl to release the hexosamines Equal amounts from the hydrolysates were analyzed for galactosamine on the amino acid analyzer with and without the addition of an inter nal standard of similarly hydrolyzed *N*-acetylga lactosamine.

*Isolation of glycopeptides of human FSH.* Hu man FSH and its subunits, viz., FSH-$\alpha$ and FSH-$\beta$ were isolated as described earlier [8]. 32 mg o purified human FSH were digested with pronase and gel-filtered as described previously [3] to ob tain glycopeptide fractions. The glycopeptide-con taining fractions were purified by high-voltage paper electrophoresis at pH 6 to isolate the $\alpha$-52 and $\alpha$-78 glycopeptides [3]. The $\beta$-7 glycopeptide was isolated from the undigested high molecular weight material from the column, which was re duced and *S*-aminoethylated in the presence of 8 M urea and refractionated on Sephadex G-50 (superfine). The peptide-containing fraction was

lyophilized and digested with 100 μg of 1% Pronase in 0.05 M Tris-HCl (pH 8.0)/1 mM $CaCl_2$. Additional aliquots of 100 μg Pronase were added at 24 and 48 h intervals and the supernatant was centrifuged and fractionated on a 1.9 × 250 cm column of Sephadex G-50 (superfine) (Fig. 2) in 0.1 M ammonium bicarbonate/5% propanol. Fractions containing glycopeptides were identified by the phenol-sulfuric acid test [9], and the β-7 glycopeptide was purified by high-voltage paper electrophoresis at pH 6.0 and/or pH 3.5.

The glycopeptides were examined for purity by high-voltage electrophoresis at pH 3.5, by thin-layer chromatography on MN-300 cellulose coated plates (Analtech) using the developing solvent pyridine/acetic acid/water (50:30:15, v/v) and visualized with a spray of 1% ninhydrin in acetone. Glycopeptide spots were eluted and subjected to acid hydrolysis and amino acid analysis to determine their hexosamine content. Amino acid sequence determination was performed on all glycopeptides.

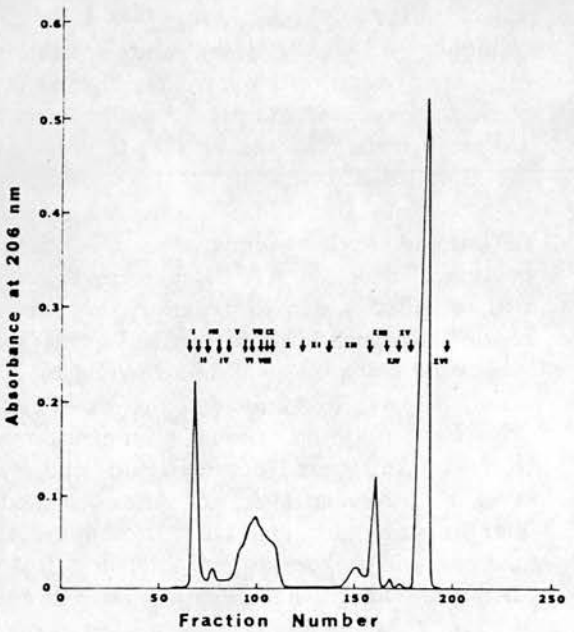



Fig. 2. Gel filtrations of the pronase digest of reduced and *S*-aminoethylated peptides on a 1.9×250 cm column of Sephadex G-50 superfine in 0.1 M ammonium bicarbonate/5% propanol. Flow rate: 12.9 ml/h; fraction volume: 4.3 ml. Fractions were pooled as indicated by arrows and lyophilized.

*Characterization of the glycopeptides.* In addition to the determination of the sequence of the monosaccharides, the isolated carbohydrate moieties were furhter studied, as follows, in order to confirm the position of GalNAc and the possible presence of terminal 'sulfated sugar' in human FSH carbohydrate moieties.

(1) The glycopeptides showing a single entity in TLC were analyzed for content of galactosamine to rule out contamination with other galactosamine-containing glycopeptides. (2) The presence of galactosamine was substantiated by amino acid analysis using internal standards as well as by a TLC separation of hexosamines. (3) The possible presence of an additional galactosamine residue in one of the side chains was examined by treatment of asialoagalactoglycopeptide with exo-α-*N*-acetylgalactosaminidase following acid hydrolysis of the glycopeptide to remove any sulfate residues, if present. (4) The possible source of GalNAc from a contaminating moiety, where galactosamine may be singly linked to threonine at position β-6, was studied by the digestion of the asialoagalacto-β-7 with the exo-α-*N*-acetylgalactosaminidase. (5) Digestions with endoglycosidases D and H were performed to verify the presence of the sequence -Man-GalNAc-GlcNAc-Asn in the inner core instead of the sequence of -Man-GlcNAc-GlcNAc-Asn. Similar digestions using a purified fetuin glycopeptide were performed to examine the activity of these enzymes on a glycopeptide with a known inner core structure of Man-GlcNAc-GlcNAc-Asn. (6) Elution behavior of labeled glycopeptides on a DEAE-Sephacel column was studied to determine the possible presence of a 'sulfate' group. The assumption was based on the interesting finding of a sulfate group on a terminal galactosamine residue as described in bovine LH by Parsons and Pierce [7].

*Carbohydrate composition of the α-52 and β-7 glycopeptides of human FSH.* The α-52 and β-7 glycopeptides were analyzed for neutral sugar, hexosamine and sialic acid content. The neutral sugar and hexosamine contents were determined by gas-liquid chromatography according to the procedures of Clamp et al. [10] and Reinhold [11]. In a typical analysis, 2 nmol glycopeptide and 10 nmol inositol, as an internal standard, were dried and dissolved in 0.5 M methanolic HCl. Methanol-

ysis was performed for 16 h at 65°C in sealed tubes under nitrogen for determination of all sugars except fucose, where methanolysis was performed for 1.5 h at 65°C. After methanolysis and acetylation were completed, the samples were dissolved in 25 μl of Sylon HTP (Supelco) to form *O*-trimethylsilyl ethers of the methyl glycosides. An appropriate aliquot of this mixture was injected into a Perkin-Elmer 900 gas chromatograph equipped with an Infotronics CRS-104 digital readout system. Chromatographic separation was achieved on a 4 mm (i.d.) × 183 cm glass column packed with Chromosorb WHP coated with 3% OV-17 polymer (Supelco). The column was equilibrated at 100°C and programmed over a temperature gradient of 6 C.deg./min to 250°C, the final temperature being held until the last peak had eluted. Peak areas were determined manually or with the integrator. The hexosamine content was also determined on a Durrum-500 amino acid analyzer following a 4 h hydrolysis of the glycopeptide in 5.7 M HCl containing 0.2% phenol at 105°C.

In addition, 5 nmol aliquots of the glycopeptides which had been hydrolyzed for 4 h were dried, redissolved in water and applied to MN-300 cellulose-coated thin-layer plates. The plates were developed in ethyl acetate/pyridine/glacial acetic acid/water (5:5:1:3) to separate hexosamines. Standard *N*-acetylglucosamine and *N*-acetylgalactosamine treated similarly, were co-chromatographed. To visualize the hexosamines, the plates were sprayed with 1% silver nitrate in acetone solution, air dried and sprayed with 0.5% KOH in methanol.

The sialic acid content of the glycopeptides was determined by the thiobarbituric acid method [12], following hydrolysis of the glycopeptide in 0.1 M HCl for 1 h at 85°C. The quantity of each monosaccharide present in the α-52 and β-7 glycopeptides was calculated as mol monosaccharide/mol glycopeptide with the asparagine residue set at 1.0.

*Enzymatic digestions of α-52 and β-7 glycopeptides with glycosidases.* Neuraminidase (EC 3.2.1.18) (Type IX, *Clostridum perfringens*) β-D-*N*-acetylhexosaminidase (EC 3.2.1.52) and β-D-galactosidase (EC 3.2.1.23) derived from *Aspergillus niger* were gifts from Dr. O.P. Bahl (State University of New York, NY). β-Galactosidase and β-D-*N*-acetylhexosaminidase were isolate from *Diplococcus pneumoniae* culture filtrates previously described [20]; α-L-fucosidase from be kidney (EC 2.3.1.51) was obtained from Boe ringer-Mannheim. The specific activity of eac enzyme was determined by reaction with nitrophenylglycosidase substrates at 37°C in eac of the following buffers at pH 4.6: 0.05 M sodiu citrate; 0.1 M ammonium acetate; 0.1 M pyridi acetate. One unit of activity was defined as th amount of enzyme which liberates 1 μmol nitrophenol/min at 37°C. Exo-α-*N*-acetylgala tosaminidase from *Clostridium perfringens* was ob tained from BRL Laboratories (Rockville, MA endoglycosidases D and H were obtained fro Miles Laboratories (Elkhart, IN).

In order to determine the sequence of th monosaccharide units in the carbohydrate moi ties, sequential enzymatic digestions using variou combinations of glycosidases were performed o the glycopeptides as described below.

Aliquots of 20 nmol each of the α-52 and β- glycopeptides were digested with α-fucosida (0.01 U, 72 h), neuraminidase (1.15 U, 72 h), galactosidase (0.01 U, 72 h), β-*N*-acetyl-hexos minidase (0.25 U, 9 days), and α-mannosida (1.02 U, 6 days). All enzymatic digestions we performed at 37°C and pH 4.6 under toluene in Dubnoff metabolic shaker [3]. Control exper ments consisted of enzyme(s) alone incubated u der the identical conditions. After the incubatio the samples were fractionated on a 0.5 × 150 c column of Sephadex G-50 superfine, equilibrate with distilled water to separate the enzyme(s residual glycopeptide, and released sugar(s). Frac tions were collected at 40 min intervals at a flo rate of 2–3 ml/h. Aliquots of the respective frac tions containing the residual glycopeptide or th released sugars were lyophilized and trimethylsily lated, and the neutral sugars were identified an quantitated by gas-liquid chromatography. Amin acid analysis was performed on acid hydrolysate for the determination of hexosamines. The amoun of sugar released was calculated as mol per mo glycopeptide with reference to the asparagine re sidue as 1.0.

*Digestion with exo-α-N-acetylgalactosaminidase* In order to examine the possibilities of a sulfate *N*-acetylgalactosamine residue on a periphera

chain as described by Parsons and Pierce in bovine TSH [7] and of an *N*-acetylgalactosamine residue being linked to a serine or threonine residue, 10 nmol asialogalacto-$\beta$-7-glycopeptide were treated with 0.5 N $H_2SO_4$ at 100°C for 9 min [6] to remove any 'sulfate' groups, if present. The glycopeptide, thus treated, was isolated and incubated with exo-$\alpha$-*N*-acetylgalactosaminidase in 0.01 M phosphate ($K^+$) buffer at pH 6.5 for 24 h at 37°C to cleave off any terminal galactosamine, if present. The enzyme digest was gel filtered through a previously calibrated 0.5 × 150 cm column of Sephadex (G-50), equilibrated and eluted in $H_2O$. The fractions were examined for the content of galactosamine by thin-layer chromatography and by analysis of 4 h acid hydrolysates on the amino acid analyzer, as described.

*Digestions with endoglycosidase-D and endoglycosidase-H.* In order to confirm the absence of the di-*N*-acetylchitobiose linkage Man-GlcNAc-GlcNAc-Asn, commonly found in glycoproteins, two aliquots of 5 nmol of the asialoagalactoaglucosamino $\beta$-7 glycopeptide were incubated with the above enzymes separately in 0.05 M citrate-phosphate buffer, pH 6.5 and 5.0, respectively, for 24 h at 37°C. The enzyme digests were applied to 0.5 × 150 cm column of Sephadex G-50 (superfine) equilibrated and eluted with distilled $H_2O$. Fractions of 1.5 ml, collected at 1 h intervals were, pooled according to absorbance at 280 and 206 nm and were analyzed for their amino acid and carbohydrate compositions.

*Digestions with glycosidases to prepare the 'inner core':* As described earlier [3], 100 nmol of the carbohydrate moiety of the $\beta$-7 glycopeptide was extensively digested with neuraminidase, galactosidase, hexosaminidase and $\alpha$-mannosidase to prepare the 'inner core'. The 'inner core' was freed of the liberated 'peripheral' sugars and enzymes by gel filtration on a 0.5 × 150 cm column of Sephadex G-50 (superfine), equilibrated and eluted with distilled $H_2O$. The fraction containing the 'inner core' was identified by its carbohydrate compositon.

*Periodate oxidation.* In order to establish the monosaccharide sequence of the 'inner core,' serial periodate oxidations were performed on aliquots of the residual glycopeptides as shown in Table II. Each oxidation was carried out with 0.05 M sodium metaperiodate in 0.05 M ammonium acetate buffer of pH 4.0 in dark for 22 h at 4°C [13]. The residual glycopeptide was separated from the reactants by gel-filtration on a 0.7 × 30 cm Bio-Gel P-2 column equilibrated with 0.05 M pyridine acetate and analyzer for carbohydrate composition.

*Alkaline degradation of human FSH subunits.* In order to determine the possible existence of sugar moieties linked to serine or threonine, alkaline degradation of human FSH-$\alpha$ and of human FSH-$\beta$ subunits was performed by incubation of 100 $\mu$g of each subunit with 100 $\mu$l 1.0 M $NaBH_4$ solution in 0.1 M NaOH at 45°C [14]. Aliquots of 20 $\mu$l were taken at intervals of 4, 8, 12, 24 and 32 h, for amino acid analysis. Threonine, serine alanine and galactosamine contents were determined in each aliquot.

*Ion-exchange chromatography on DEAE-Sephacel.* The objective of this experiment was to examine the elution profile for possible content of sulfate in the glycopeptide. Intact and asialoglycopeptides were labeled by reductive methylation using $H^{14}CHO$ [15] and repurified by gel filtration on Sephadex G-50 (superfine) columns. [$^{14}$C]Dimethyl glycopeptides were mixed with NeuAc 2 → 3 [$^3$H]lactitol [21] as the internal standard and chromatographed on a DEAE-Sephacel column. Glucose 6-sulfate (Sigma) was separately chromatographed under similar conditions and its elution position was determined by anthrone reaction.

## Results and Discussion

*Galactosamine content of human FSH, LH, TSH, bovine LH and human CG (Table I; Fig. 3)*

As shown in Fig. 3, the retention volume of the internal standard *N*-acetylgalactosamine was the same as the *N*-acetylgalactosamine in the unknown samples, confirming that we were measuring galatosamine, and not an artifact. Further, as shown in Table I, the known amounts of galactosamine added to the unknown samples were quantitatively recovered.

Human FSH, LH, their subunits, and human TSH of pituitary origin purified by different laboratories have galactosamine present in their carbohydrate moieties. The carbohydrate moieties of bovine pituitary LH and TSH also contain

TABLE I

GALACTOSAMINE CONTENT OF HORMONES

| | nmol GalNAc found | Sample + internal standard | GalNAc content (residues/mol [a]) |
|---|---|---|---|
| Human FSH | 1.2 | 3.0 [b] | 2.7 |
| Human FSH (NPA) (AFP-4822-B) [d] | 1.0 | 2.6 [c] | 3.5 |
| Human FSH (LER-1978-3) [e] | 1.5 | 3.6 [c] | 2.6 |
| Human FSH-α | 1.4 | 3.5 [b] | 1.6 |
| Human FSH-α (NPA) (LER-1815-2) | 1.2 | 3.4 [c] | 1.6 |
| Human FSH-β | 0.7 | 2.3 [c] | 0.6 |
| Human FSH-β (NPA) (AFP-4911-B) | 0.5 | 2.0 [c] | 0.5 |
| Human LH | 1.5 | 3.7 [b] | 2.0 |
| Human LH (NPA) (AFP-4345-B) | 1.7 | 3.2 [c] | 2.2 |
| Human LH-α (NPA) (AFP-2442-C) | 0.6 | 2.5 [c] | 2.3 |
| Human LH-β (NPA) (AFP-141-B) | 0.6 | 2.5 [c] | 1.1 |
| Bovine LH (NPA) (Batch 1941-1) | 1.2 | 2.8 [b] | 2.1 |
| Bovine LH (NPA) (NIAMDD-bLH-4) | 0.6 | 2.5 [c] | 2.1 |
| Human TSH | 1.7 | 3.5 [b] | 3.1 |
| Human TSH (NPA) (AFP-4370-B) | 1.1 | 2.6 [c] | 3.9 |
| Bovine TSH (NPA) (NIAMDD-bTSH-9) | 1.0 | 2.7 [c] | 4.1 |

[a] The following molecular weights were used for the calculation: human FSH, 32600; the subunits, 16000; human LH, human TS bovine LH and bovine TSH 27000.

[b] 2 nmol GalNAc were added to the sample.

[c] 1.7 nmol GalNAc was added as internal standard.

[d] Hormones received from National Pituitary Agency (NPA), MD.

[e] hFSH prepared by L.E. Rechert, Jr., The Albany Medical College, Albany, NY.

galactosamine. Human CG, a placental glycoprotein and human LH, which have as much as 80% homology in their amino acid sequences, differ in their galactosamine content. However, differences in the carbohydrate structures of glycoproteins with identical polypeptide sequences are known. In fact, even the single carbohydrate unit of highly purified preparations of ovalbumin has been shown to be a mixture consisting of nine different structures attached to the same position of the polypeptide backbone. Similarly, α-1-acid glycoprotein from human serum has at least 19 carbohydrate structures at the five attachment sites along the polypeptide chain. Unlike polypeptide chains, the synthesis of which is under direct control of the genetic code, the oligo- and polysaccharides are not primary gene regulated products; therefore, their synthesis often results in microheterogeneity [17].

The galactosamine content of the three human FSH preparations (Table I) indicate two residues in the α-subunit and one residue in the β-subunit [3]. As shown in Table I, the galactosamine co tent of the human FSH-α and β preparations fro our laboratory as well as of those supplied by t NPA are similar, attesting to the purity of t preparations used in the present study [1 Galactosamine was also found to be present human LH and human TSH, prepared by us well as by other investigators.

*Isolation of glycopeptides*

The human FSH-α-52 glycopeptide, which w isolated and purified after the first pronase dige of the human FSH [3], consisted of five amin acid residues, Gln-Lys-Asn(CHO)-Val-Thr, corr sponding to the sequence of residues 50 to 54 human FSH-α [1]. The gel filtration of the secon pronase digest of the reduced and *S*-aminoeth lated fraction is shown in Fig. 2. Human FSH-β was isolated by high-voltage paper electrophores from the fraction (V-X). The isolated glyc peptides migrated as a single entity in analytic paper electrophoresis at pH 3.5, indicating th

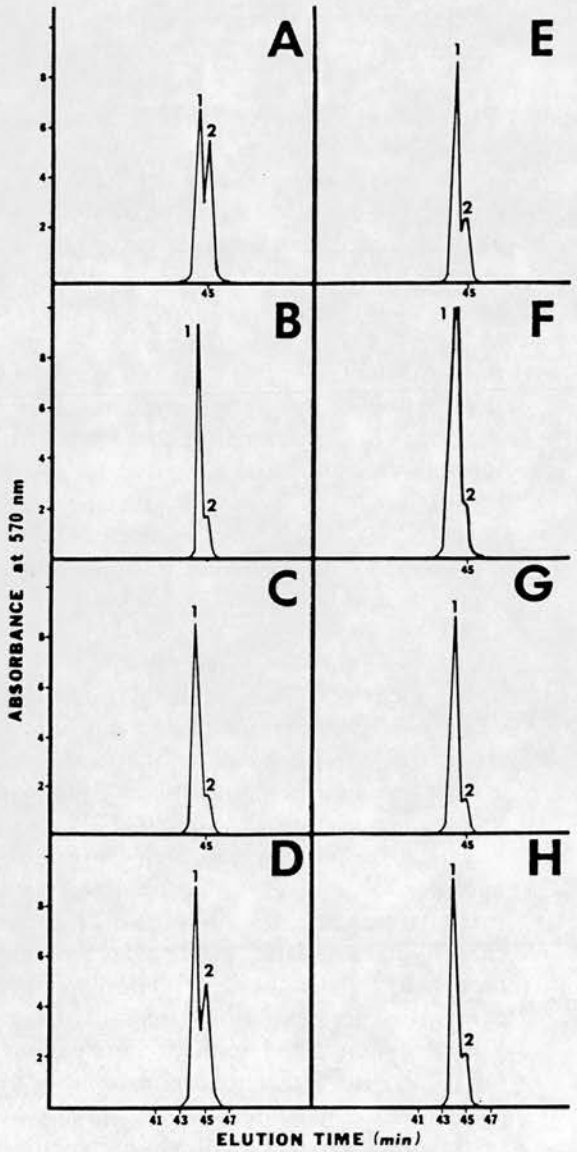



Fig. 3. Determination of galactosamine in 4-h hydrolysates of pituitary hormones on the amino acid analyzer: (A) GlcNAc : GalNAc standard (1 : 1); (B) GlcNAc : GalNAc standard (5 : 1); (C) human FSH-α; (D) human FSHα + GalNAc standard (2.5 nmol); (E) bovine LH; (F) human LH; (G) human FSH-α-52 glycopeptide; (H) human TSH. Elution times: GlcNAc (1), 44 min; GalNAc (2), 45 min.

homogeneity of the glycopeptide which was further validated by the integral ratio of the amino acids in the glycopeptide. The human FSH-β-7 glycopeptide consisted of three amino acid residues with the sequence Thr-Asn(CHO)Ile corresponding to the residues 6–8 in human FSH-β subunit [2].

The thin-layer chromatography of β-7 glycopeptide showed a single spot, which after elution and analysis contained galactosamine, attesting that the β-7 glycopeptide was pure and did not contain any other contaminating glycopeptide. Separation of hexosamines, by thin-layer chromatography, after a 4 h hydrolysis of β-7 glycopeptide, also distinctly revealed the presence of galactosamine in the β-7 glycopeptide.

*Digestion of human FSH-α-52 moiety with specific glycosidases*

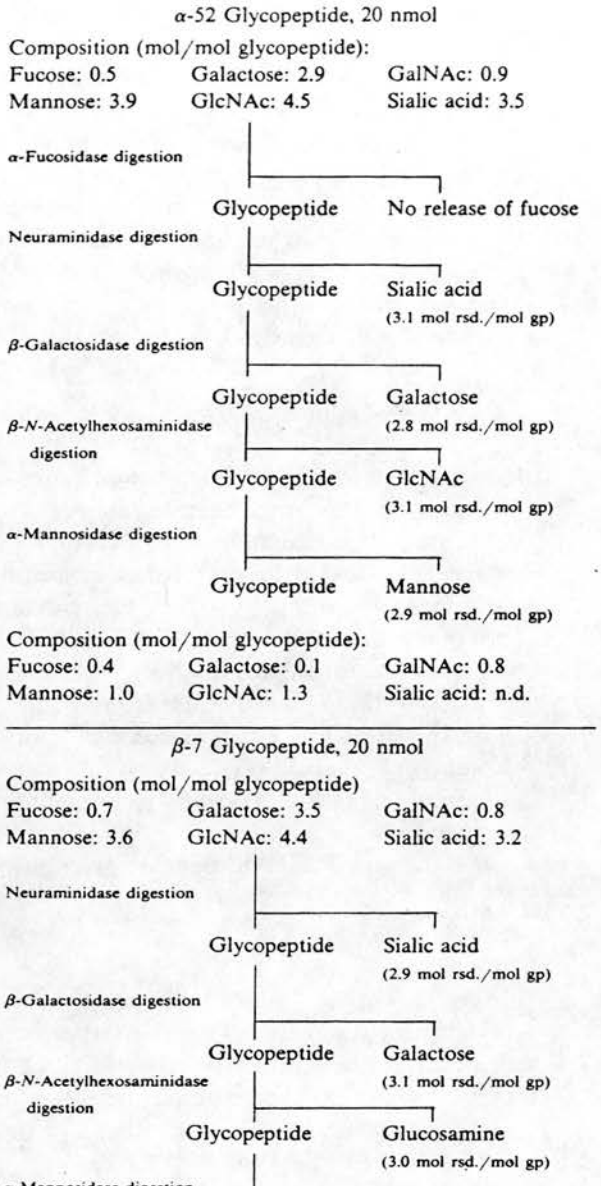
The carbohydrate composition and the sequence of this glycopeptide are presented in Scheme I. The neuraminidase digestion liberated three sialic acid residues. Further digestion of the glycopeptide with β-galactosidase, β-*N*-acetylhexosaminidase and α-mannosidase released three residues each of galactose, GlcNAc, and mannose, indicating the presence of three peripheral chains with the sequence of monosaccharide units as follows: sialic acid → galactose → *N*-acetylglucosamine → mannose. It should be noted that this sequence is similar to that of human FSH-α-78 LH-α-52, and TSH-α-52 reported previously [3].

*Digestion of human FSH-β-7 glycopeptide with specific glycosidases*

As shown in Scheme I, the digestions of the carbohydrate moiety attached to the asparagine residue 7 of human FSH-β showed three peripheral chains of the sequence, sialic acid → galactose → *N*-acetylgalactosamine → mannose, similar to that of human FSH-α-52.

*Digestion of human FSH-β-7 glycopeptide with exo-α-N-acetylgalactosaminidase*

The enzyme digest was applied to a Sephadex G-50 (superfine) column in order to separate *N*-acetylgalactosamine, if present, from the residual glycopeptide. Pooled fractions did not contain free galactosamine indicating the absence of GalNAc in a terminal position either in a peripheral sidechain or in a GalNAc-Thr linkage. The acid hydrolysis would have removed any 'sulfate' group, if present on the galactosamine so that the GalNAc




Scheme I. Sequential glycosidase digestion of human FSH-α-52 and human FSH-β-7 glycopeptides. Mol monosaccharide/mol glycopeptide was determined by setting Asn = 1.0. rsd., released; gp, glycopeptide. n.d., not detectable.

residue would have been susceptible to the enzym action.

*Digestion of human FSH-β-7 glycopeptides wit endoglycosidases D and H*

The essential sugar residue for the action of th endoglycosidase D is an unsubstituted α-mannosy residue linked to the innermost β-mannosyl re sidue by 1–3 linkage [18]. The endoglycosidase H catalyzes the cleavage of the linkage $(GlcNAc)_2$ $(Man)_m$-GlcNAc-X-GlcNAc-Asn. After incuba tion with these enzymes, there was no evidence o the cleavage of the glycopeptide moiety, indicatin that the β-7 glycopeptide did not contain th linkages specific for these enzymes. In fact, thes results would be expected with the sequence o Man-GalNAc-GlcNAc-Asn proposed by us. O the other hand, the fetuin glycopeptide with th known Man-GlcNAc-GlcNAc-Asn was cleaved.

*Alkaline degradation of the subunits of human FSH*

The presence of GalNAc in the inner core regio of the α-78 glycopeptide of human FSH previousl studied [3] presented a unique carbohydrate se quence for an asparagine linked glycopeptide. Al kaline degradation of human FSH using 0.1 M $NaBH_4$ did not reveal Ser/Thr-linked carbo hydrate moieties [3,10]. In order to confirm furthe the existence of only asparagine-linked carbohydrate moieties in the α- and β-subunits o human FSH, alkaline degradation was performe using stronger conditions than used previously The results indicated neither an increase in alanin nor a decrease in galactosamine, serine or threonin content, thereby confirming the absence of serine or threonine-linked carbohydrate moieties.

*Periodate oxidation*

The compositions of the residual 'inner core' o the α-52 and of the β-7 carbohydrate moietie revealed one molecule each of mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine and fucose (Table II). From the composition, the se quence of the inner core appears to be similar t that of human FSH-α-78. The periodate oxida tions of the residual inner core regions of the α-52 and β-7 glycopeptides indicated the loss of fucos and mannose in the first oxidation and the loss o *N*-acetylgalactosamine in the second, thus con

firming the sequence of the inner core region to be:

Man→GalNAc→GlcNAc→Asn
Fucose

*Ion-exchange chromatography on DEAE-Sephacel (Fig. 4)*

The elution pattern of human FSH-β-7 indicated microheterogeneity due to variation in the sialic acid content (Fig. 4a) since after desialylation the major portion eluted in essentially two peaks (Fig. 4b). Hence, 90% of the glycopeptide did not contain 'sulfate' as judged from its elution prior to neuraminlactitiol. The minor peak (fractions 79–90, Fig. 4b) eluted in the position of the control glucose 6-sulfate suggests that 10% of the glycopeptide may contain a 'sulfate' residue possibly on a second galactosamine residue in one of the peripheral side-chains. This is also confirmed by the treatment of the desialylated β-7 with acid, prior to ion-exchange chromatography on DEAE-Sephacel. Acid treatment would have removed the sulfate group; and a decrease in the amount of the minor peak would have been expected, as shown in Fig. 4c. Analyses of the major peak revealed a GlcNAc:GalNAc ratio of 4:1 and of the minor peak of 2:1. According to the monosaccharide sequence shown in Fig. 1, one GalNAc residue in the inner core moiety will give a ratio of GlcNAc:GalNAc of 4:1. If there were one additional GalNAc in the peripheral side-chain instead of a GlcNAc, then the ratio of GlcNAc:GalNAc would be 1.5:1. If the additional GalNAc in the peripheral side-chain was a substituent of a galactose residue, the GlcNAc:GalNAc ratio will be 2:1. If the GalNAc were present only in the

TABLE II

PERIODATE OXIDATION OF THE HUMAN FSH-α-52 AND HUMAN FSH-β-7 'INNER CORE' GLYCOPEPTIDES

| Carbohydrate | Composition (mol/mol glycopeptide) [a] | | |
|---|---|---|---|
| | Prior to oxidation [b] | 1st oxidation | 2nd oxidation |
| α-52 | | | |
| Fucose | 0.4 | 0.0 | 0.0 |
| Mannose | 1.0 | 0.5 | 0.0 |
| GalNAc | 0.8 | 0.8 | 0.4 |
| GlcNAc | 1.3 | 1.3 | 1.3 |
| β-7 | | | |
| Fucose | 0.7 | 0.0 | 0.0 |
| Mannose | 1.1 | 0.1 | 0.0 |
| GalNAc | 0.9 | 0.9 | 0.3 |
| GlcNAc | 1.2 | 1.1 | 1.1 |

[a] Mol monosaccharide/mol glycopeptide determined by setting Asn value = 1.0.

[b] After digestion with glycosidases.

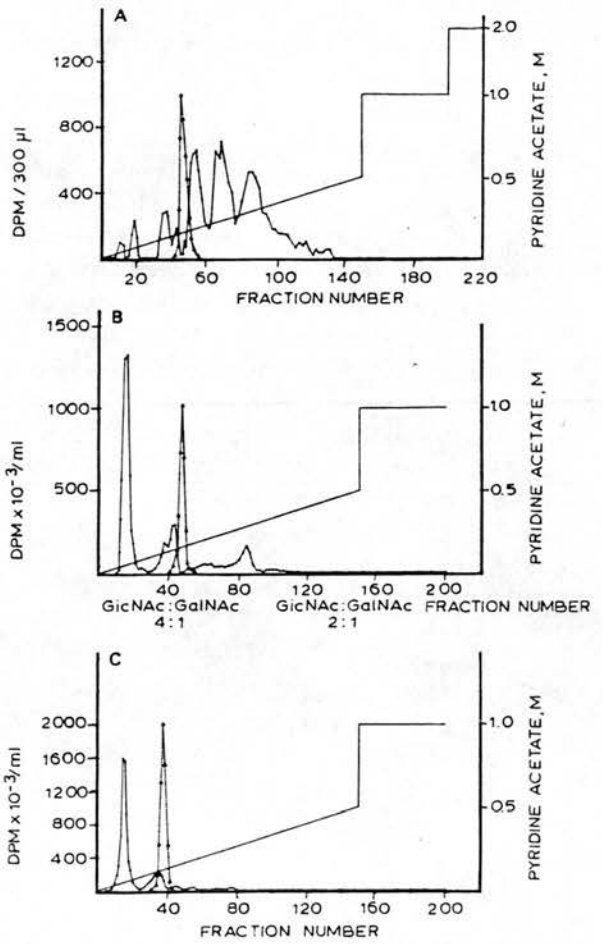



Fig. 4. Ion-exchange chromatography on DEAE-Sephacel (A) of untreated [$^{14}$C]dimethyl β-7 glycopeptide (●——●); (B) of neuraminidase treated [$^{14}$C]dimethyl β-7 glycopeptide (●——●); and (C) of acid treated asialo [$^{14}$C]dimethyl β-7 glycopeptide. NeuAc→[$^{3}$H]lactilol (△···△) was used as an internal standard. The column was eluted with a linear gradient of 0.01–0.5 M pyridine/actic acid (1 M, 2 M), at the rate of 20 ml per h. Fractions of 2.0 ml were collected and 1.0 ml aliquots analyzed for radioactivity.

peripheral chain and were a substituent for a galactose, then GlcNAc:GalNAc ratio would be 5:1. If it were a substituent of a GlcNAc then the ratio again would be 2:1. Therefore, it appears that the major peak contains an *N*-acetylgalactosamine the inner core; whereas, the minor peak may contain either two residues of *N*-acetylgalactosamine or only one in the peripheral chain.

In conclusion, our results indicate that three of the four carbohydrate moieties of human FSH are identical and have the monosaccharide sequence shown in Fig. 1. It is of interest to note that in the closely related glycoprotein human CG, all the four asparagine-linked carbohydrate moieties are identical [5,19]. The carbohydrate moieties of human CG, however, contain only two peripheral chains per moiety [5] as compared to three of human FSH.

## Acknowledgements

This study was supported by the National Institutes of Health (Grant No. HD 06543). Thanks are due to Dr. O.P. Bahl (State University of New York, Buffalo, NY) for supplying glycosidases and to Mr. Ken Miller for performing some of the amino acid analysis.

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# ANNUAL MEETING

## he Society for Complex Carbohydrates

## September 25-27, 1980

# ABSTRACTS

61 DETECTION OF GLYCOPHORIN-LIKE GLYCOPROTEINS ON THE SURFACE OF CULTURED HUMAN CELLS. V. P. Bhavanandan and A. L. Barsoum. Dept. of Biol. Chem., The M.S. Hershey Med. Ctr., The PA State Univ., Hershey, PA 17033.

In previous studies we have isolated and characterized mucin-type glycopeptides from mouse and human melanoma cells. These glycopeptides have clusters of oligosaccharides of the type NeuNAc → Gal → (NeuNAc) → GalNAc linked to serine and or threonine (Bhavanandan et al. (1977) Biochemistry 16, 4426) suggesting an apparent similarity to glycophorin. We now report the interaction of anti-glycophorin antibodies with various cultured cells. Anti-serum to highly purified glycophorin was prepared in rabbits. Human melanoma cells (HM7), human breast cells (HBL-100) and two lines of human breast cancer cells (MCF-7 and MDA-231) showed medium to very strong cell surface fluorescence pattern after staining with rabbit anti-glycophorin $F(AB)_2$ and FITC-conjugated goat anti-rabbit $F(AB)_2$. Cells treated with pre-immune rabbit $F(AB)_2$ and FITC-conjugated goat anti-rabbit $F(AB)_2$ or with FITC-conjugated goat anti-rabbit $F(AB)_2$ alone did not show detectable fluorescence.

Immunodiffusion, immunoelectrophoresis and affinity chromatography on anti-glycophorin IgG-Sepharose 4B of detergent extracts of metabolically labeled cultured cells gave further evidence for the presence of glycophorin-like components in these cells. (This work was supported in part by U.S.P.H.S. Grants CA17686 and CA15483.)

# FIRST ANNUAL CONGRESS FOR HYBRIDOMA RESEARCH

## PRODUCTION OF MONOCLONAL ANTIBODIES TO GLYCOPHORIN

A.L. Barsoum, V.P. Bhavanandan and E.A. Davidson, Department of Biological Chemistry, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033

Glycophorin, a transmembrane sialoglycoprotein of the human erythrocytes, carries on its N-terminal region, the MN blood group determinants and influenza virus receptors. In a previous communication (V.P. Bhavanandan and A.L. Barsoum: Annual meeting, The Society for Complex Carbohydrates, 1980), we showed the presence of Glycophorin-like molecules on various cultured human cells using polyclonal rabbit antibodies in an indirect immunofluorescent test. The present report describes the production of monoclonal antibodies to glycophorin by fusion of spleen cells from BALB/c mice immunized against purified glycophorin and the non-secreting variant of the P3 myeloma line. The hybrids were screened for antibody production using a solid-phase binding radioimmunoassay. Positive wells (4/480) were subcloned by limiting dilution. After screening the subclones by complement dependent hemolysis in gel, we selected a clone, Glyco N/P3/1, which reacts with the N-terminal portion of glycophorin. Further investigations of the nature of the monoclonal antibody produced by this clone and its reactivity to human erythrocytes and cultured human cells are in progress. (This work was supported in part by U.S.P.H.S. Grant CA15483).

**57** INTERACTION OF GLYCOPROTEINS WITH WHEAT GERM AGGLUTININ-SEPHAROSE OF VARYING CONCENTRATIONS OF LECTIN PER MILLILITER GEL. Kiyoshi Furukawa, John E. Minor and V.P. Bhavanandan, Dept. of Biol. Chem., The M.S. Hershey Medical Center, The PA State Univ., Hershey, PA 17033

In previous studies on wheat germ agglutinin (WGA) we had observed that the interaction of glycoproteins was influenced partly by the density of lectin molecules on the gel beads (J. Biol. Chem. 254, 4000, 1979). In the present study we prepared a series of WGA-Sepharose 4B gels containing 0.08, 0.53, 0.95, 1.28, 3.08 and 7.30mg WGA per ml gel. Equal volumes of the gels were packed in columns and tested for their ability to bind *in vitro* labelled glycoproteins and glycopeptides. It was found that whereas the mucin-type glycoproteins having a high density of sialic acid bound virtually completely to all the WGA-Sepharose gels tested, the serum-type glycoproteins bound very poorly or not at all to the gels with low concentrations of lectin. To determine the interaction of membrane sialoglycoproteins with WGA-Sepharose gels, studies were conducted with plasma membranes isolated from rat liver and Morris Hepatoma 7777. The membrane glycoproteins were solubilized with 0.5% NP-40 and labelled on sialic acid by treatment with $NaIO_4$ and $NaB^3H_4$. The [$^3$H] sialoglycoproteins, after purification by chromatography on a column of DEAE-Sepharose, were tested for their ability to interact with the WGA-Sepharose gels. It was found that 38.5%, 51.0% and 58.4% of the hepatoma membrane sialoglycoproteins bound to WGA-Sepharose gels having 0.10, 0.53 and 3.08mg WGA per ml gel, respectively. In contrast only 9.0%, 36.7% and 48.7% of the liver membrane sialoglycoproteins bound to the same gels.

# Influences of anionic polysaccharides on DNA synthesis in isolated nuclei and *in vitro*; correlation of observed effects with properties of the polysaccharides

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Running title: Effect of glycosaminoglycans on DNA synthesis

Key words: DNA synthesis, heparin effect, histones, hepatoma

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FOOTNOTE

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SUMMARY

The basis of the differential effect of anionic polysaccharides on replicative DNA synthesis in liver and hepatoma cell nuclei was investigated. The differential effect of heparin was lost when more than 40% of its sulfate was removed. DNA synthesis in liver nuclei was optimally stimulated by heparin of molecular weight 22,600 and sulfate to hexosamine ratio 2·42 but inhibited by heparin of molecular weight 4300 and sulfate to hexosamine ratio 2·35. A heparin fragment, (molecular weight 2800 and sulfate to hexosamine ratio 1·81) prepared by partial nitrous acid treatment was a potent inhibitor of DNA systhesis in hepatoma nuclei. There was no significant difference in the rate of entry of heparin or its subfractions into either liver or hepatoma nuclei. In both cases less than 15% of added polysaccharide entered the nuclei and only about 4.5% was found associated with the chromatin. The influence of the anionic polysaccharides on DNA synthesis was correlated with their ability to complex with histones as determined by relative light scattering in a laser nephelometer. The relative light scattered on mixing with histones (H1, H2A + H3, H4) was high for DNA synthesis stimulators (heparin, dextran sulfate); medium for DNA synthesis inhibitors (chondroitin 4- and 6-sulfates, (heparan sulfate) and low for non-effectors (keratan sulfate, hyaluronic acid). Heparin and chondroitin sulfate H, which at low concentrations stimulate DNA synthesis in liver nuclei, inhibited in vitro DNA synthesis by calf thymus DNA polymerase α at all concentrations. The inhibition of in vitro DNA synthesis by anionic polysaccharides was not simply due to electrostatic interactions.

Glycosaminoglycans were previously considered to be secretory products found in the extracellular matrix. It is now clear that in addition to this localization, these molecules are also present in significant quantities at the cell surface [1], in the plasma membrane [2], cytosol [3], nucleus [4,5] and mitochondria [6]. For example, Prinz et al. [7] showed that of the glycosaminoglycans synthesized by primary cultures of hepatocytes only 13% were secreted whereas 30% became associated with the cell surface and the balance remained within the cells.

These observations have prompted several investigators to ask whether these polyanionic macromolecules, in addition to their accepted extracellular (structural and organizational) functions, may have regulatory or modulating roles on intracellular events. Circumstantial evidence has been obtained indicating the possibility of involvement of acidic polysaccharides in replicative DNA synthesis [8-11], RNA synthesis [9,12,13], protein synthesis [14], regulation of development of sea urchin embryos [15], cell adhesion [16,17] and growth control [18,19].

Previously, we have established beyond doubt the presence of anionic glycoconjugates in animal cell nuclei and characterized some of these nuclear components [4,5]. Results of other workers have confirmed and extended these findings [20-22]. Subsequently we have turned our attention to an examination of the effects of natural and synthetic (model) anionic glycoconjugates on DNA synthesis.

In a previous paper we reported the differential response of heparin and chondroitin sulfate H on endogenous DNA synthesis in isolated normal and cancer cell nuclei [23]. The present study focuses on the molecular basis for the action of anionic saccharides on DNA synthesis. The results

show that the molecular size, charge (degree of sulfation) and possibly special structural features of the polysaccharides are important factors in determining the type of influence (inhibition or stimulation) exerted. These studies also provide evidence that the stimulatory and the inhibitory effects of certain polysaccharides can be correlated with their ability to interact with histones and DNA polymerase, respectively. It is the ultimate purpose of our studies to (i) contribute to the understanding of the biological functions of glycosaminoglycans, (ii) obtain information on their possible role in regulation of DNA replication in vivo in animal cells, (iii) isolate an anionic polysaccharide molecule which would inhibit DNA synthesis in cancer cell nuclei, but have no or minimal effect on normal cell nuclei.

## MATERIALS AND METHODS

Male Buffalo rats were obtained from Simonsen Laboratories (Gilroy, CA). Livers from 12 week-old animals were used for nuclei isolation. Morris hepatoma 7777 [24] was maintained by bilateral implantation into the thigh muscle of the rats.

The procedures for the isolation of nuclei and assay of endogenous DNA synthesis in nuclei were as described previously [23]. When aphidicolin [25,26] was included in the assay mixture at a concentration of 300μg per ml, DNA synthesis was inhibited by 84%. Addition of N-ethylmaleimide (5-10mM) also inhibited DNA synthesis by 80-95%.

DNA Synthesis (in Vitro) [27]. Calf thymus DNA polymerase α purchased from Worthington (Freehold, NJ) was used in these experiments. Assays were done in triplicate in 50 mM Tris-HCl (pH 7.4) - 8 mM $MgCl_2$ - 1 mM dithothreitol - 30 mM KCl containing 15% glycerol, 400 μg per ml bovine serum albumin,

0.1 mM dATP, dCTP and dGTP, 0.06mM (5μCi) [$^3$H] dTTP (New England Nuclear, Boston, MA) and 100μg of calf thymus DNA which was activated by a combination of freezing and thawing followed by digestion with DNase I according to Kornberg and Gefter [28]. DNA polymerase α (0.1 - 0.2 units) and, where indicated, various concentrations of glycosaminoglycans as their sodium salts were added and the reaction mixture (total volume 0.5 ml) was incubated for 3 hours at 37°. The reaction was terminated by the addition of cold 10% trichloroacetic acid -0.1 mM dTTP - 10 mM sodium pyrophosphate. The precipitate was collected, washed, dissolved and the radioactivity determined as described [23].

Histone-Glycosaminoglycan Interaction. Calf thymus histone (Calbiochem, La Jolla, CA) was fractionated into histones H1, H2A + H3, H2B and H4 by chromatography on a column of Bio-Gel P-60 [29]. The histone fractions and glycosaminoglycan samples were dissolved in 0.1 M Tris-HCl (pH 7.0) - 0.15 M NaCl containing 5% (w/v) polyethylene glycol 6000 and 15 mM $NaN_3$ and the solutions filtered through 0.2μ Millipore filters. Appropriate volumes (2-40μl) of the glycosaminoglycan solutions (500μg per ml) were placed in test tubes (10 x 75 mm), diluted with buffer (also filtered through Millipore filters) to 0.985 ml and 15μl of histone solution (200μg per ml) added. The solution was mixed by gentle end over end mixing and the relative light scattering measured by laser nephelometry using a Hyland Laser Nephelometer PDQ. The complex formation appeared to be instantaneous, therefore, measurements were made within one hour of adding the histone. Incubations up to 24 hours had no further influence on light scattering provided the solutions were mixed before measurements.

Partial Desulfation of Heparin. In order to prepare heparin with differing

sulfate content, heparin (200 mg) was converted to its pyridine salt and subjected to partial solvolysis at 80° with dimethyl sulfoxide containing 10% water [30]. Aliquots (20 mg) were removed at various times and treated with acetic anhydride to N-acetylate the free amino groups of the hexosamine residues [31]. The products were analyzed for hexosamine and sulfate content. The time of treatment in minutes and the percent sulfate removed are as follows: 0.1, 4.7; 0.2, 18.8; 0.5, 38.6; 5, 55.6; 15, 62.5; 30, 66.1; 45, 67.2; 60, 67.5; 120, 71.1; 180, 79.1 and 240, 83.8.

Preparation of Isotopically Labelled Heparin. Heparin (20 mg) was tritiated according to Hatton et al. [32] by treatment with 5 mCi of $NaB^3H_4$ (10 Ci/mmol), (Research Products International Corp., Mount Prospect, Ill) in 200µl of 0.2M borate buffer (pH 8.4). After incubation at room temperature for 3 hours, 300µl of $NaBH_4$ solution (10mg per ml in borate buffer) were added and after a further 1.5 hours, the reaction mixture was extensively dialyzed and lyophilized. The specific activity of the [$^3$H] heparin preparations varied from $1.0 \times 10^7$ to $2.1 \times 10^7$ dpm per mg. $^{14}$C-labelled heparin was also prepared by reductive methylation using $H^{14}CHO$ and $NaCNBH_3$ [33].

Partial Nitrous Acid Treatment. Heparin was partially fragmented by exposure to limiting amounts of nitrous acid followed by dilute sulfuric acid [34,35]. Heparin (200mg) was dissolved in 4 ml of 0.05 M $NaNO_2$ in 0.2 M sodium citrate (pH 1.5), incubated for 30 min at 0° and the pH then adjusted to 8.0 by addition of pyridine. The mixture was chromatographed on a column of Bio-Gel P-10 (2.0 x 155cm) using 0.1 M pyridine/acetic acid as eluant. Aliquots of the column fractions were assayed for uronic acid [36] and the positive fractions were combined and lyophilized. The

recovered material was dissolved in distilled water, the pH adjusted to 1.9 with dilute sulfuric acid and incubated for 30 min at 50°; the reaction mixture was then neutralized and chromatographed on Bio-Gel P-10 as above.

Heparin samples were obtained from Sigma (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). Semi-purified heparin used in the preparation of the heparin fractions of different molecular weight was kindly donated by Scientific Protein Laboratories (Waunakee, WI) and Hepar Industries (Franklin, Ohio). Heparin fractions of varying molecular size [37] were provided by Dr. Erik Holmer, Kabi Vitrum, Stockholm (Sweden). Sodium pentosan sulfate [14] was a gift from Dr. Halse, Benechemie, Munchen (W. Germany); and carrageenan samples were donated by Dr. D.A. Rees, Unilever Research, (United Kingdom). Aphidicolin was a gift from Dr. A. H. Todd, ICI, (United Kingdom). Other glycosaminoglycans used were as reported previously [23]. Dextran sulfates 500, 40 and 8 of molecular weights 500,000, 40,000 and 8,000 respectively were obtained from Sigma (St. Louis, MO) and cellulose sulfate from Aldrich (Milwaukee, Wis). All natural and synthetic polysaccharides were tested for protease and nuclease activities [23]; none was detected in the samples used. Deoxynucleotide triphosphates were purchased from Sigma (St. Louis, MO) and adenosine triphosphate from Calbiochem (La Jolla, CA).

The DNA content of nuclei was determined by the method of Burton [38] using calf thymus DNA as standard. Hexosamine was assayed after hydrolysis (6N HCl, 100°C, 12h). The hydrolysates were dried, redissolved in buffer and aliquots containing 0.5 to 2.0 nmoles of hexosamine analyzed on a Dionex amino acid analyzer using fluoropa for detection. Sulfate was determined by the barium chloranilate assay [39] and neutral sugars by the phenol-$H_2SO_4$ assay [40].

## RESULTS

Differential effect of sulfated polysaccharides on DNA synthesis.

In Table I, the effects of various highly sulfated polysaccharides on DNA synthesis in nuclei isolated from rat liver and hepatoma are shown. The maximum degree of stimulation of DNA synthesis obtainable in liver nuclei, as well as the polysaccharide to DNA ratio required to stimulate maximally, varied widely. Polysaccharides with a low degree of sulfation (chondroitin sulfate H and carrageenans) showed only slight stimulation (215 to 270%) at rather high polysaccharide to DNA ratios (0.62 and 0.75 respectively). The polysaccharides with high degree of sulfation, and high (Dextran sulfate 500 and cellulose sulfate) or low (pentosan sulfate) molecular weights also showed only slight stimulation (231 to 275%). However, these were active at lower polysaccharide to DNA ratios (0.14 to 0.33) as compared to the less sulfated polysaccharides. The highest stimulations were shown by polysaccharides with a high degree of sulfation but of intermediate molecular weights (heparin, dextran sulfates 40 and 8).

In contrast to the wide variation in their ability to stimulate DNA synthesis in liver nuclei, the influence of these polysaccharide on DNA synthesis in hepatoma nuclei was more consistent. All the highly sulfated polysaccharides (sulfate/ disaccharide 2.77 to 3.90) with the exception of dextran sulfate 500 gave 50% inhibition of DNA synthesis at polysaccharide to DNA ratios of 0.07 to 0.09. Larger amounts (polysaccharide to DNA ratios of 0.18 to 0.40) of the polysaccharides with a low degree of sulfation were required to obtain the same degree of inhibition.

The observation that the low molecular weight (about 3000) pentosan sulfate stimulated DNA synthesis in liver nuclei and inhibited DNA synthe-

sis in hepatoma nuclei was of particular interest. To understand the role of size and degree of sulfation within a single class of polysaccharide, the differential effect of subfractions of heparin on DNA synthesis was investigated. The influence of the heparin subfractions of varying molecular weight (Figure 1) on DNA synthesis in isolated nuclei from liver and hepatoma is detailed in Table II. At a ratio of heparin to DNA of 0.42, subfractions I, II (maximum stimulation, 6-fold), III, IV and V (lowest stimulation, 2.4 fold) stimulated DNA synthesis in liver nuclei. At higher ratios of heparin to DNA (>0.9), these five subfractions inhibited DNA synthesis. The lowest molecular weight fraction, VI, inhibited DNA synthesis in liver nuclei increasingly with increasing concentrations.

All six heparin subfractions consistently inhibited DNA synthesis in hepatoma nuclei (Table II). In the molecular weight range 6,700 to 31,000 (subfractions V to I), the degree of inhibition was inversely related to molecular size and independent of the heparin to DNA ratio. The optimum stimulation of liver nuclear DNA synthesis was shown by heparin of molecular weight 22,600, and the optimum inhibition of DNA synthesis in hepatoma nuclei was by the 6700 molecular weight heparin. The dissimilar molecular size requirements for optimum stimulation and optimum inhibition is of considerable interest.

Results identical to those reported above were also obtained with heparin subfractions of varying molecular weight obtained by fractionation of heparin from Scientific Protein Laboratories and those donated by Dr. Erik Holmer [37].

Similar studies with three fractions of dextran sulfates obtained by chromatography on Bio-Gel P-60 gave analogous results.

Effect of degree of sulfation (anionic character) of heparin on DNA synthesis. We reported that the degree of inhibition of DNA synthesis in liver and hepatoma nuclei by chondroitin sulfates and heparan sulfate was directly correlated with the sulfate to hexosamine ratio [23]. DNA synthesis in hepatoma nuclei was likewise inhibited by the partially desulfated heparin fractions. At all the heparin to DNA ratios tested, the degree of inhibition was directly correlated with the sulfate content (Figure 2).

Heparin with sulfate to hexosamine ratio of more than 1.7 exhibited a differential effect on DNA synthesis in liver nuclei which was dependent on the ratio of heparin to DNA. At ratios below 0.9, DNA synthesis was stimulated while above this ratio DNA synthesis was inhibited (cf. results obtained with native heparin [23]). At a fixed heparin to DNA ratio, the degree of stimulation decreased linearly with the degree of sulfation (sulfate/hexosamine 2.7 to 1.7) (Figure 2). Heparin fractions having sulfate to hexosamine ratios less than 1.7, showed only inhibition of DNA synthesis in liver nuclei.

Effects of heparin fragments obtained by partial nitrous acid treatment on DNA synthesis. To study the combined effect of size and charge, the influence of heparin fragments prepared by partial nitrous acid treatment on DNA synthesis was determined. The estimated molecular size, the sulfate to hexosamine ratio and the influence of these randomly cleaved fractions on DNA synthesis in hepatoma nuclei are summarized in Table III.

The most effective inhibitors of DNA synthesis were fractions B and C having estimated molecular weights of 2,800 and 1,600, respectively. Fraction B also has a low sulfate to hexosamine ratio. Thus, we have iden-

tified a heparin fragment having about fourteen saccharides and 1.8 sulfate residues per hexosamine which is a potent inhibitor of DNA synthesis in isolated hepatoma nuclei. In agreement with the result obtained with heparin subfraction VI (Table II), this fragment had no stimulatory activity on DNA synthesis in liver nuclei at any concentration; at low concentrations it had no effect and at high concentrations it inhibited DNA synthesis.

Entry of heparin into nuclei and association with chromatin. The time course of the entry of heparin into liver and hepatoma nuclei and the relationship of the entry to the concentration of DNA in the incubation mixture are illustrated in Figure 3. It is clear that there is no significant difference in the rate of entry of heparin into liver or hepatoma nuclei and that saturation levels of heparin in both cases are reached after 2 to 3 hours. Thus, in all experiments assessing the influence of anionic polysaccharides on DNA synthesis in intact nuclei, incubation was for a period of 3 hours.

The nmoles of [$^3$H] heparin and three [$^3$H] heparin fractions of varying molecular weight which were associated with liver or hepatoma nuclei are given in Table IV. The results indicate that there is no significant difference in the total amount of heparin associated with the liver and hepatoma nuclei; in both cases about 1 nmole of heparin per 100 µg DNA was found. There was also no correlation between the molecular size of heparin and its ability to enter (or associate with) the nuclei, suggesting a passive entry mechanism.

The distribution of the [$^3$H] heparin within the nucleus was also studied. Nuclei, after incubation with [$^3$H] heparin as described, were

successively extracted twice with 75 mM NaCl - 24 mM EDTA (pH 8.0), twice with 50 mM Tris-HCl (pH 8.0), and finally with water adjusted to pH 8.0 to solubilize the chromatin [42]. Each extraction was done by mixing for 2 min followed by centrifugation at 1,000 x g for 10 min. This procedure was employed rather than the high ionic salt extraction [43] which is liable to dissociate heparin-chromatin complexes. Of the total [$^3$H] heparin associated with the nuclei 62.5%, 30.4% and 7.1% were recovered in the buffer extracts, water extract (chromatin) and residue, respectively. In comparison the distribution of DNA in these fractions was 17%, 78% and 5%. Thus, about 30% and 78% of the recovered [$^3$H] heparin and DNA respectively, were present in the chromatin fraction. It can be calculated that 0.3 ± 0.09 nmoles (4.0 ± 1.3 μg) of [$^3$H] heparin was associated with 100μg of DNA in chromatin.

Interaction of glycosaminoglycans with histones. The observed stimulation of DNA synthesis in nuclei isolated from normal tissues may be attributable to the interaction of anionic polysaccharides with histones, thereby removing template restrictions [44,45]. Experimental support for this was provided by the observation that addition of histones to the incubation mixture nullifies the stimulatory effect of heparin on DNA synthesis [46]. Additional correlation was sought between the effect of various glycosaminoglycans on DNA synthesis and their ability to interact with histones. Complex formation of the polysaccharides with histones was determined by laser nephelometry, a technique which has been used for the measurement of antigen-antibody complexes [47], glycoprotein-lectin interactions [48] and circulating immune complexes [49] and serum glycosaminoglycans [50].

Based on the results shown in Figure 4 we examined the interaction of

various glycosaminoglycans at 20μg per ml with unfractionated histones or histones H1, H2A + H3, H2B, and H4 at 3μg per ml (Figure 5). Heparin and dextran sulfate 40, which have differential effects on DNA synthesis, showed the highest light scattering on interaction with unfractionated histones as well as histones H1, H2A + H3 and H4. Chondroitin 4- and 6-sulfates, heparan sulfate and N-desulfated heparin, all of which inhibit DNA synthesis, showed moderate light scattering with these histones. Hyaluronic acid, keratan sulfate, and desulfated heparin, which have no effect on DNA synthesis, showed the lowest light scattering with the same histones. The complex formation between the glycosaminoglycans and histones H2B was poor and could not be correlated to the effects on DNA synthesis.

Similar results were obtained when the heparin subfractions varying in molecular size (Figure 1) were tested for complex formation with histones. The complex forming ability of subfractions I to V with histones (unfractionated, H1 and H2A +H3) roughly paralleled the degree of stimulation of DNA synthesis (see Table II). For example, fractions II and V which were the most and least stimulatory for DNA synthesis showed the highest and lowest light scattering, respectively, when mixed with unfractionated histones and histones H1 or H2A + H3.

Effect of glycosaminoglycans on DNA Synthesis in Vitro. These experiments were carried out to assess the effect of glycosaminoglycans on DNA polymerase α. DNA synthesis, assayed by incorporation of [$^3$H]dTTP, was linear with time (up to the 3 hours tested) and with increasing concentrations of DNA polymerase (tested up to 0.2 units per 100μg DNA per 0.5ml (Figure 6a and c). At an enzyme concentration of 0.1 units per 0.5ml, the reaction

reached saturation at 100μg DNA per 0.5ml (Figure 6b).

The effects of various glycosaminoglycans on polymerase activity during a 3 hour incubation are summarized in Table V. The most potent inhibitors were heparin and heparin subfractions II to V (data not presented), followed by chondroitin sulfate H. It is of interest that heparin inhibited in vitro DNA synthesis at all levels tested, even if added 15 min after the onset of the reaction. In contrast, in liver nuclei these polysaccharides stimulate DNA synthesis (Tables I and II). Heparan sulfate, chondroitin-4 and 6-sulfates, and N-desulfated heparin also inhibit DNA synthesis and in this group the degree of inhibition parallels the degree of sulfation and was directly dependent on the polysaccharide to DNA ratio. Hyaluronic acid, dermatan sulfate, keratan sulfate and desulfated heparin had no significant effect on in vitro DNA synthesis which is in agreement with their influence on DNA synthesis in isolated nuclei [23].

## DISCUSSION

The process of DNA replication and the mechanism of its regulation is very complex [51]. Conflicting views have been presented regarding the cytoplasmic and/or nuclear localization of DNA polymerase α, the replicative enzyme in mammalian cells [52]. Recently, immunochemical methods were used to demonstrate that the bulk of this enzyme is present in the perinuclear region of the cytoplasm [53]. veer Reddy and Pardee [54] have shown that although DNA polymerase α is virtually all cytoplasmic in $G_1$ phase cells, in S-phase, this enzyme is assembled into a replication apparatus in the nucleus. Thus, the level of DNA replication observed in isolated nuclei is probably an indication of the proportion of S-phase cells in the original population.

We used the method of Lynch et al. [55] for the isolation of nuclei, which yielded nuclear preparations suitable for replicative DNA synthesis studies [56]. Further, the conditions used by us to assay DNA synthesis were similar to those previously demonstrated to carry out replicative DNA synthesis rather than repair [46,57,58]. The inhibition of DNA synthesis by aphidicolin and N-ethylmaleimide, believed to be specific for DNA polymerase $\alpha$, is further evidence that replicative DNA synthesis was being measured. Using the identical procedure for nuclei isolation and assay, the level of [$^3$H]dTTP incorporation into DNA was lowest in rat liver, high in regenerating liver and highest in hepatoma nuclei, data which support the replicative rather than repair process. The system employed is also well suited to study the effects of nucleocytoplasmic interactions on DNA replicaton with endogenous templates.

The present study confirms that the stimulatory effects shown by several highly sulfated polysaccharides in liver nuclei can be attributed to the abolishing of template restriction. Although the polyanionic character of these molecules plays a crucial role in this, the degree of stimulation was not directly correlated to the degree of sulfation (Tables I and II, Figure 2). These results, as do previous data, confirm that the observed effects are not simply due to electrostatic interactions. In addition to the overall charge of the polysaccharide, factors such as charge distribution, conformational change with alteration of charge and perhaps the degree of ionization may all play a role in the interaction with template and/or DNA polymerases. In addition to an optimum charge, the importance of an optimum polysaccharide size for stimulating DNA synthesis was also clearly demonstrated. For example, a heparin subfrac-

tion with molecular weight 9500 and sulfate to hexosamine ratio 2.31 stimulated DNA synthesis in liver nuclei four-fold, whereas the fraction with molecular weight 4300 and a sulfate to hexosamine ratio 2.35 did not (Table II).

The inhibitory influence of the various polyanions tested appears to be due to the direct interaction with the polymerase. Once again, the effects observed are not simply due to electrostatic interactions although these do play a role. Thus, in in vitro DNA synthesis, keratan sulfate with a sulfate to hexosamine ratio of 1.43 was a very poor inhibitor but heparan sulfate with a sulfate to hexosamine ratio of 1.31 inhibited DNA synthesis by 50%. Polysaccharides with higher sulfate content were generally better inhibitors in intact nuclei than those with a lower degree of sulfation (Figure 2). The effect of polysaccharide molecular size on inhibition of DNA synthesis in intact nuclei was striking. Intermediate size polysaccharides were more potent inhibitors than either the very large or very small molecular weight species. Thus, pentosan sulfate (M W 3,000 and $SO_4$/disaccharide 3.64) and heparin subfraction V (M W 6,700 and $SO_4$/disaccharide 2.19) were both very effective inhibitors. A similar molecular weight dependence of the anticoagulant activity of heparin has been demonstrated by several investigators [35,37,59]. We failed to detect any significant difference in the ability of the heparin fractions of varying molecular size to enter the nuclei (Table IV). Therefore, the observed differences in the inhibition of nuclear DNA synthesis by these subfractions are unlikely to be due to different rates of entry. The secondary structure, shape and conformations of the saccharides are probably involved in their interaction with the DNA polymerase.

The influence of glycosaminoglycans on DNA synthesis can be correlated to their ability to complex with histones; most strikingly with H1, but also to lesser extents with H2A + H3, H4 and mixture (Figure 5). Histone H1 gave very high light scattering (>180) with DNA synthesis stimulators (heparin, dextran sulfate 40 and heparin fractions I to IV), intermediate light scattering (100 to 136) with DNA synthesis inhibitors (chondroitin 4- and 6-sulfates, heparan sulfate, N-desulfated N-reacetylated heparin and heparin subfraction VI) and very low light scattering (<30) with non-effectors of DNA synthesis (keratan sulfate, hyaluronic acid and desulfated heparin). The interaction of the polyanionic glycosaminoglycans and the cationic histones is complex and will be influenced by factors such as salt concentration and pH, among others. We have therefore measured the light scattering under conditions (pH and ionic concentrations) as close as possible to those used in the DNA synthesis assay. The relative light scattering is probably a reflection of both the size and number of complexes which are formed but at the present it is not possible to distinguish between the contributing effects of these two factors. The ability of heparin, dextran sulfate 40 and heparin subfractions I to IV to interact strongly with histones H1 and H2A + H3 may be crucial in removing template restriction and thereby stimulating DNA synthesis. These saccharides caused only inhibition when <u>in vitro</u> DNA synthesis was assayed, further proving that the stimulation in whole nuclei is due to their ability to remove histones from DNA in chromatin.

Since five out of six of the heparin subfractions still show differential effects on DNA synthesis, it is unlikely that different species in the unfractionated heparin is responsible for the differential effect. The

rates of entry of heparin into liver and hepatoma nuclei were similar, therefore the differential effects on DNA synthesis are not simply ascribable to different rates of entry. It is likely that the observed differences are due to high and low binding affinities of heparin to histones and DNA polymerase $\alpha$, respectively. In the DNA synthesis assays although large amounts of polysaccharide were used, (polysaccharide to DNA (w/w) ratios of 0.1 to 1.0) only a minor portion is associated with the chromatin. Further, it is possible that only a fraction of this may be responsible for the observed effects on DNA synthesis. This suggests that the small levels of glycosaminoglycans detected in the cell nucleus [5,21] or a small portion of the cytoplasmic glycosaminoglycans entering the nucleus could be sufficient to affect nuclear DNA replication. Thus, a physiological role for glycosaminoglycans or sialoglycoproteins in in vivo DNA replication needs to be further investigated.

In the studies reported here, heparin and semi-synthetic sulfated polysaccharides were used as models to understand the influence of polyanionic saccharides on DNA synthesis. The glycosaminoglycans most likely to be physiologically relevant are the heparan and chondroitin sulfates. The presence of these glycosaminoglycans in the nucleus, cytoplasm and cell surface has been reported; although no data on their size and degree of sulfation are available. Our previous [23] and present results have shown that subtle changes in the degree of sulfation and/or size of these glycosaminoglycans can drastically change their influence on DNA synthesis and thus play a role in the control of DNA synthesis during differentiation and development. In view of the reported alterations in the sulfation level of heparan sulfate in malignant cells [60-63], the

importance of this in the loss of growth control of transformed cells should be considered. It should also be pointed out that even though heparin has not been found in cells other than mast cells, heparin-like molecules have been detected in sea urchin embryos [64] and cultured endothelial cells [65].

In conclusion, the results reported are consistent with our previous finding that heparin and other highly anionic polysaccharides play a dual role in DNA synthesis in whole nuclei. Both the extent of sulfation and molecular size of the polysaccharides had an effect on the type and degree of influence on DNA synthesis. These studies support our contention that the lack of heparin stimulation of DNA synthesis in cancer cell nuclei is due to the already maximal template activity. The isolation of a heparin subfraction which at low concentrations is capable of inhibiting DNA synthesis in hepatoma nuclei but not normal liver nuclei is of pharmacological interest. It may be possible to exploit these polysaccharide fractions in suppressing the division of cancer cells without affecting normal cells.

## ACKNOWLEDGEMENTS

We wish to thank Dr. E.A. Davidson for his support, interest and many valuable suggestions during the course of this work. We are grateful to the following for their generous gifts of samples: Mr. John Bard, Scientific Protein Laboratories (Waunakee, WI); Dr. Margaret Haines, Hepar Industries (Franklin, OH); Dr. Halse, Benechemie (Muchen, W. Germany); Dr. Erik Holmer, KABI Vitrim (Stockholm, Sweden); Dr. David Rees, Unilever Research (United Kingdom) and Dr. A.H. Todd, ICI (United Kingdom). We also thank Mrs. Filomena Cramer for secretarial assistance. This investigation

was supported by research grant CA 15483 from the National Institutes of Health, United States Public Health Service and by an American Cancer Society Institutional grant.

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FIGURE LEGENDS

Figure 1: Fractionation of heparin by gel filtration.

Heparin (200mg) was applied to a column (2.5 x 93 cm) of Sephadex G-100 and the column eluted at a rate of 10 ml per h with 50mM Tris-HCl, pH 8.0 containing 0.1 M NaCl. Four milliliter fractions were collected and aliquots analyzed for uronic acid. The material was pooled in six fractions (I to VI) as indicated, and recovered by dialysis using 3500 dalton cut off membrane followed by lyophilization. Each fraction was then individually rechromatographed on the same column and the material eluting in the area indicated by horizontal arrows recovered as above. Only the rechromatographic profiles of fractions II, III, IV and V are illustrated for clarity. The elution positions of [$^{3}$H] porcine submaxillary mucin ($V_o$) and [$^{14}$C] glucose ($V_t$) are indicated.

Figure 2: Effect of sulfate content of heparin on endogenous DNA synthesis in liver and hepatoma nuclei. Endogenous DNA synthesis in nuclei was assayed [23] in the presence of varying concentrations of partially desulfated heparin fractions. The results illustrated are for the effect of 54µg heparin per 100µg liver nuclear DNA (o----o) and of 45µg heparin per 100µg hepatoma nuclear DNA (•——•). Different concentrations showed similar effects, however, quantitative differences were present.

Figure 3: Kinetics of entry of [$^{3}$H] heparin into nuclei isolated from rat liver (o) and Morris hepatoma 7777 (•).

[$^{3}$H] Heparin (specific activity $10^{7}$ DPM per mg) was incubated with isolated nuclei under conditions identical to those used

for the DNA synthesis assay except that [$^3$H]dTTP was replaced by unlabelled dTTP. After incubation for the selected time, the nuclei were sedimented by centrifugation (1000 x g), washed 3 times with 0.15 M NaCl buffered with phosphate (pH 7.2) and twice with 0.5% Triton X-100 in the same buffer. The latter wash was expected to remove the outer nuclear membrane [41]. The radioactivity in the washes and in the pellet (after solubilization in 2% sodium dodecyl sulfate) was determined by scintillation counting.

Figure 4: Complex formation between histone and glycosaminoglycans as measured by relative light scattering. Details of the measurement of relative light scattering are described in "Materials and Methods". Panel (a) shows the effect of increasing amounts of glycosaminoglycans on the light scattering by a fixed amount (3μg per ml) of histones, Panel (b) shows the effect of increasing amounts of histones on the light scattering by fixed amount (5μg per ml) of various glycosaminoglycans: heparin (●), chondroitin-6-sulfate (o), N-desulfated N-reacetylated heparin (x), keratan sulfate (■), desulfated heparin (▲), dermatan sulfate (▼), heparan sulfate (△) and hyaluronic acid (□).

Figure 5: Complex formation between glycosaminoglycans and histones H1, H2A+H3, H2B and H4 as determined by relative light scattering. Details of the experiment are described in "Materials and Methods". The results obtained with dextran sulfate 40 were similar to that of heparin.

Figure 6: Kinetics of in vitro DNA synthesis using calf thymus DNA polymerase α.

[$^3$H]dTTP incorporation into activated calf thymus DNA was determined as described in "Materials and Methods".

Table I: Differential Effect of Sulfated Polysaccharides on Endogenous DNA Synthesis in Isolated Nuclei From Rat Liver and Hepatoma

| Sulfated Polysaccharide | Kav[a] | Sulfate / Disaccharide | Liver Nuclei: Maximum Stimulation % Control Activity (Polysaccharide)[b] / DNA | Hepatoma Nuclei: 50% Inhibition (Polysaccharide)[c] / DNA |
|---|---|---|---|---|
| Heparin | 0.22 | 2.77 | 400(0.31)[c] | (0.09)[c] |
| Dextran Sulfate 500 | 0.00 | 3.66 | 231(0.20) | (0.24) |
| Dextran Sulfate 40 | n.d | 3.90 | 520(0.52) | (0.07) |
| Dextran Sulfate 8 | 0.71 | 3.70 | 525(0.80) | (0.08) |
| Cellulose Sulfate | 0.03 | 3.08 | 275(0.14) | (0.07) |
| Pentosan Sulfate | 1.00 | 3.64 | 271(0.33) | (0.08) |
| Chondroitin Sulfate H | n.d | 1.65 | 270(0.62) | (0.18) |
| Carrageenan (Type IV) | 0.00 | 2.04 | 229(0.75) | (0.20) |
| Carrageenan (Type V) | n.d | 1.30 | 215(0.63) | (0.40) |

[a] Kav estimated by gel filtration on Bio-Gel P-60.

[b] µg polysaccharide per 100 µg DNA in 1 ml incubation mixture.

[c] At least five different polysaccharide to DNA ratios were tested in each case and the experiments were repeated. The ratios showing the maximum stimulation of DNA synthesis in liver nuclei and 50% inhibition of DNA synthesis in hepatoma nuclei are reported in parentheses.

Table II: Effect of Heparin Subfractions on Endogenous DNA Synthesis in Isolated Nuclei From Rat Liver and Hepatoma[a]

| | | | | [$^3$H]dTTP Incorporated into DNA in 3h Percent Control Activity | |
|---|---|---|---|---|---|
| | $K_{av}$[b] | Molecular Weight[b] | $\frac{\text{Sulfate}}{\text{GlcNH}_2}$ | Normal Liver[c] | Morris Hepatoma[d] 7777 |
| Heparin (unfractionated) | 0.29 | 14000 | 2.51 | 470 | 20.8 |
| Fraction I | 0.06 | 31000 | 2.59 | 491 | 97.9 |
| II | 0.15 | 22600 | 2.42 | 617 | 52.5 |
| III | 0.28 | 14600 | 2.68 | 519 | 48.5 |
| IV | 0.40 | 9500 | 2.31 | 418 | 20.7 |
| V | 0.50 | 6700 | 2.19 | 241 | 14.5 |
| VI | 0.64 | 4300 | 2.35 | 48.7 | 30.4 |

[a] similar results were obtained with subfractions of heparin from Scientific Protein Lab and heparin fractions provided by Dr. E. Holmer

[b] Kav and molecular weight estimated by gel filtration on a calibrated column of Sephadex G-100.

[c] Heparin/DNA(w/w) = 0.42

[d] Heparin/DNA(w/w) = 0.40

Table III: Effect of Heparin Fragments Obtained by Partial Nitrous Acid Treatment on Endogenous DNA Synthesis in Nuclei Isolated from Morris Hepatoma 7777

| Subfractions[a] | Sulfate / $GlcNH_2$ | Kav[b] | [$^3$H]dTTP Incorporated into DNA in 3h: Percent Control Values[c] |
|---|---|---|---|
| Heparin | 2.51 | 0 | 8.4 |
| Fraction A | 2.16 | 0.01 | 84.6 |
| B | 1.81 | 0.38 | 6.6 |
| C | 2.24 | 0.65 | 7.9 |
| D | 2.12 | 0.71 | 12.2 |
| E | 1.69 | 0.75 | 23.9 |
| F | 1.71 | 0.82 | 33.3 |
| G | 0.42 | 0.91 | 105 |

[a] Subfractions prepared as described in the text.

[b] Kav's were estimated by gel filtration on a column of BioGel P10 which was calibrated with glycopeptides of known molecular weights.

[c] Heparin/DNA (w/w) = 0.45

Table IV: Entry of [$^3$H] Heparin and Its Fractions Into Nuclei Isolated From Rat Liver and Morris Hepatoma 7777

| | Molecular Weight | Specific Activity (X10$^6$DPM/mg) | nmol. [$^3$H] Heparin or its fractions associated with 100 µg DNA after incubation for 3h | |
|---|---|---|---|---|
| | | | Liver Nuclei | Hepatoma Nuclei |
| Heparin | 14000 | 9.1 | 1.12 | 0.79 ± 0.18(2)[a] |
| Fr. I | 21000 | 11.9 | 1.16 ± 0.14(2) | 0.85 ± 0.40(3) |
| Fr. II | 7300 | 17.6 | 1.02 ± 0.04(2) | 1.31 ± 0.21(2) |
| Fr. III | 4000 | 14.2 | 0.83 | 1.16 |

[a] Number of experiments is denoted in parentheses.

Table V: Effect of Glycosaminoglycans on *In Vitro* DNA Synthesis Using Calf Thymus DNA Polymerase $\alpha$[a]

| Glycosaminoglycan | Sulfate/$GlcNH_2$ | [$^3$H]dTTP incorporated into DNA Percent Control Values |
|---|---|---|
| Heparin | 2.46 | 23.4 |
| N-Desulfated N-Reacetylated Heparin | 1.17 | 59.7 |
| Desulfated Heparin | 0.42 | 95.8 |
| Heparan Sulfate | 1.31 | 47.9 |
| Chondroitin Sulfate H | 1.65 | 44.0 |
| Chondroitin-6-Sulfate | 1.25 | 75.1 |
| Chondroitin-4-Sulfate | 1.24 | 78.5 |
| Dermatan Sulfate | 1.11 | 83.6 |
| Keratan Sulfate | 1.43 | 93.0 |
| Hyaluronic Acid | - | 99.3 |

[a] Conditions of assay are described in Materials and Methods. The effects of different concentrations of glycosaminoglycans were tested. The data presented are that obtained with 5µg of glycosaminoglycans per 100µg DNA in 0.5ml reaction mixture containing 0.1 unit of DNA polymerase $\alpha$.

Figure 1. : Furukawa and Bhavanandan -

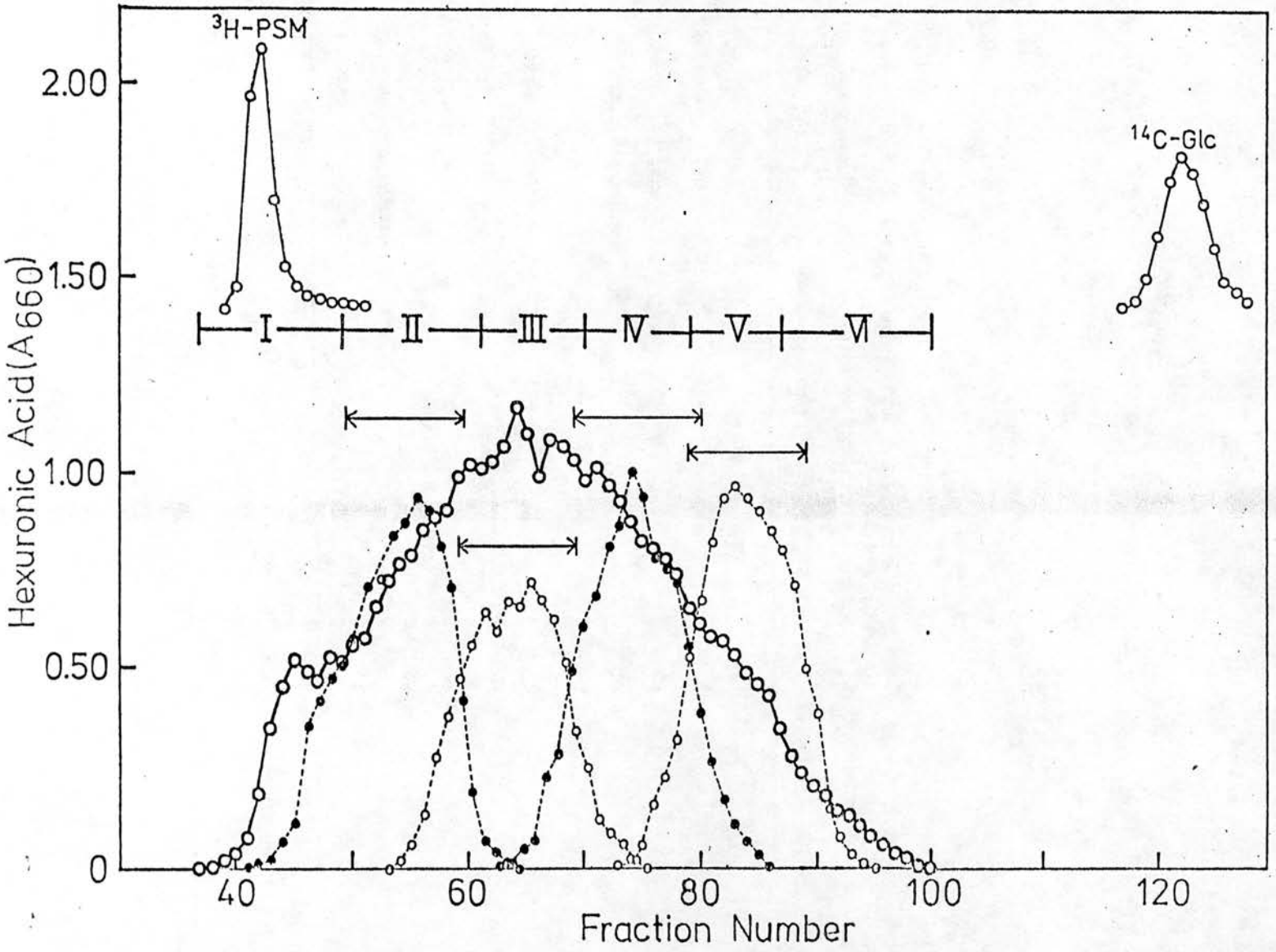
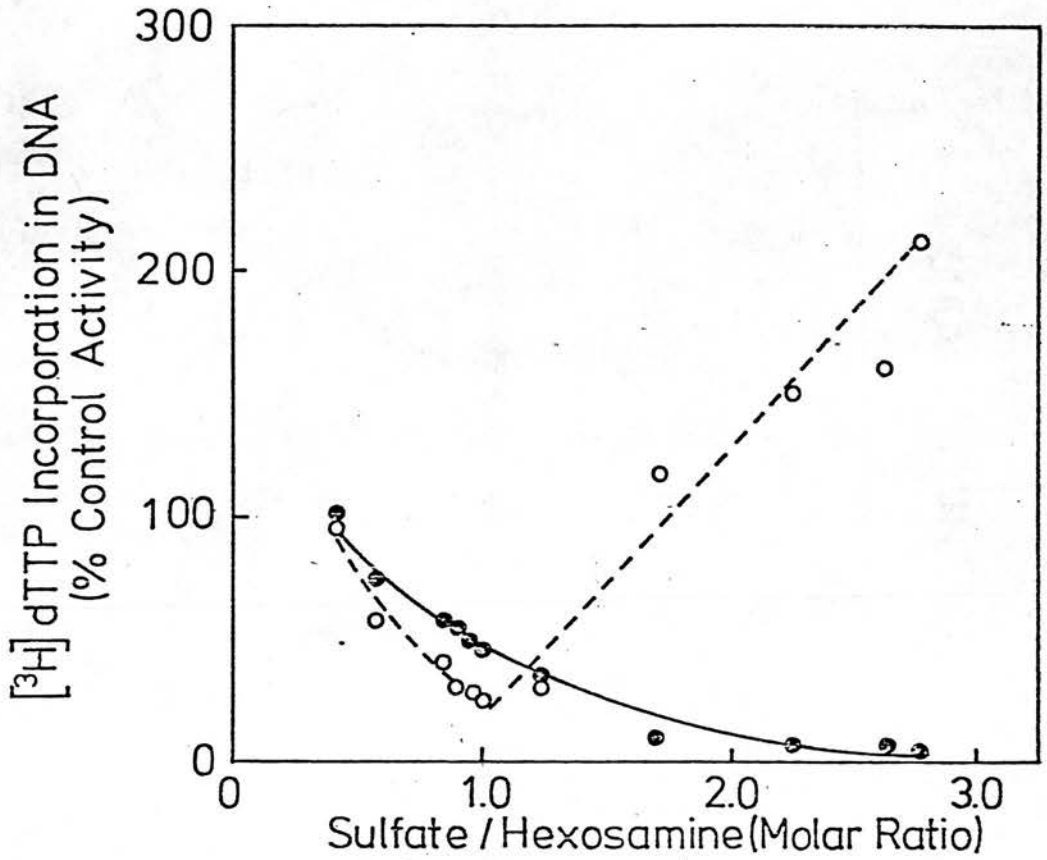
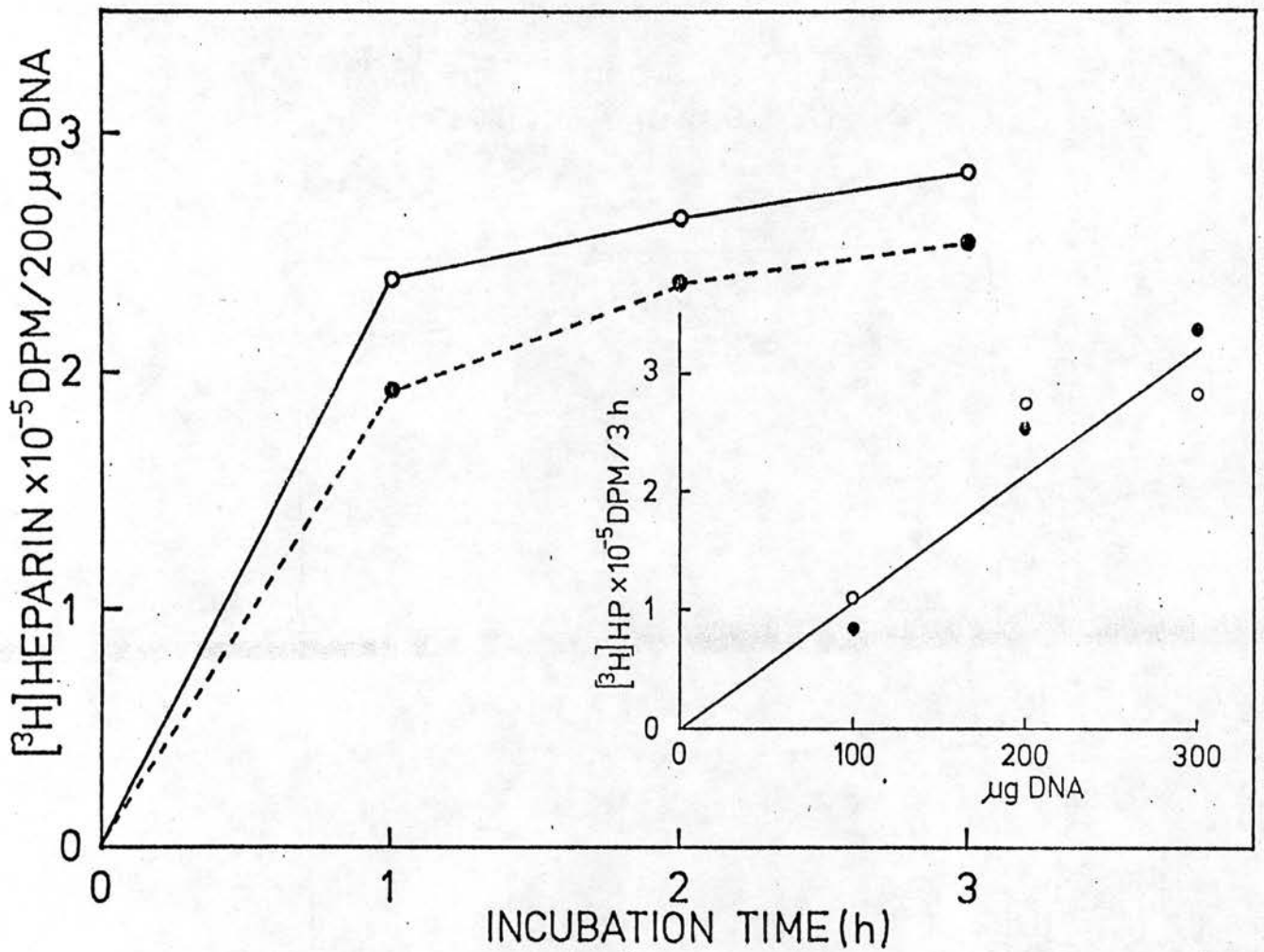
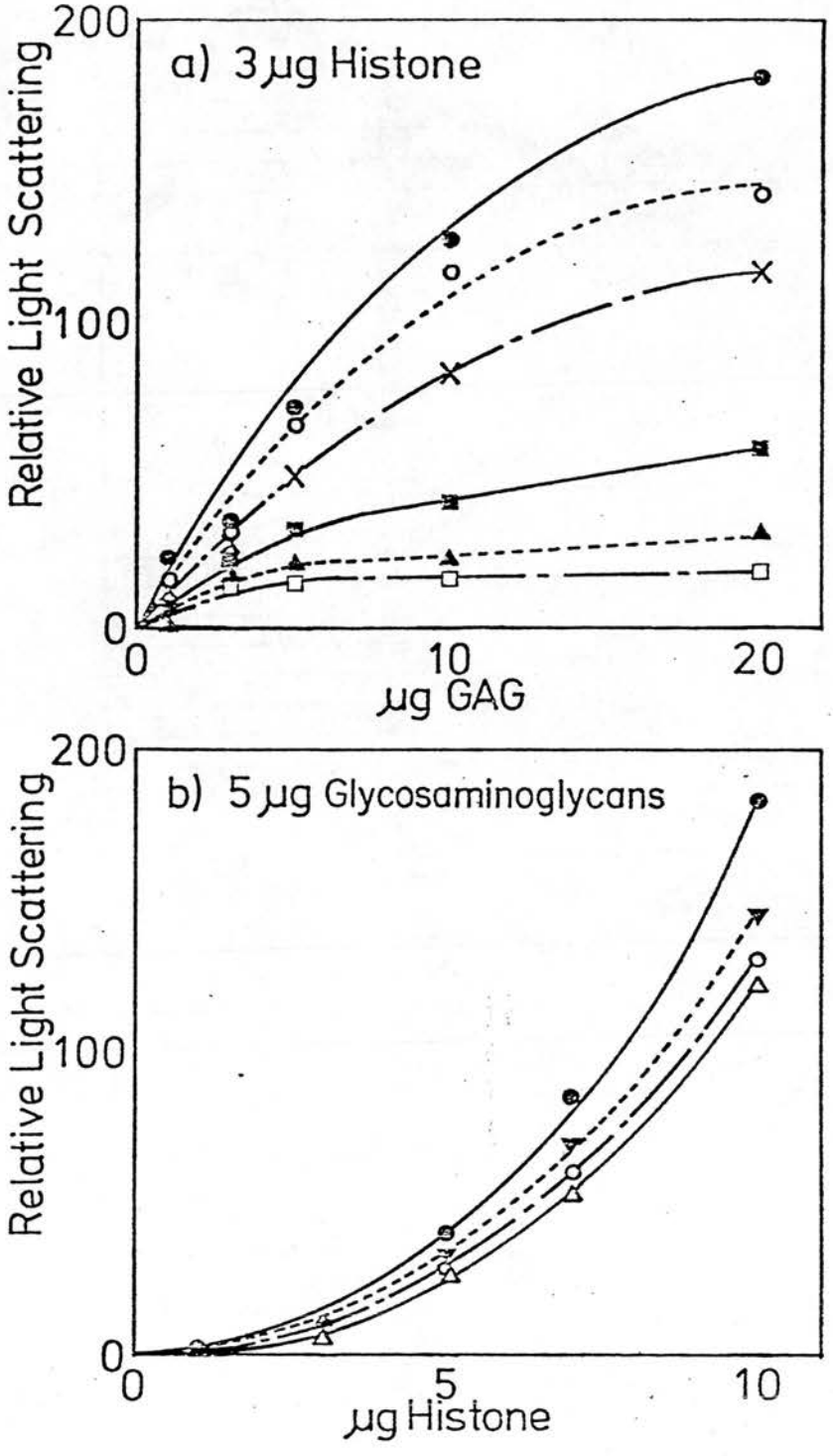


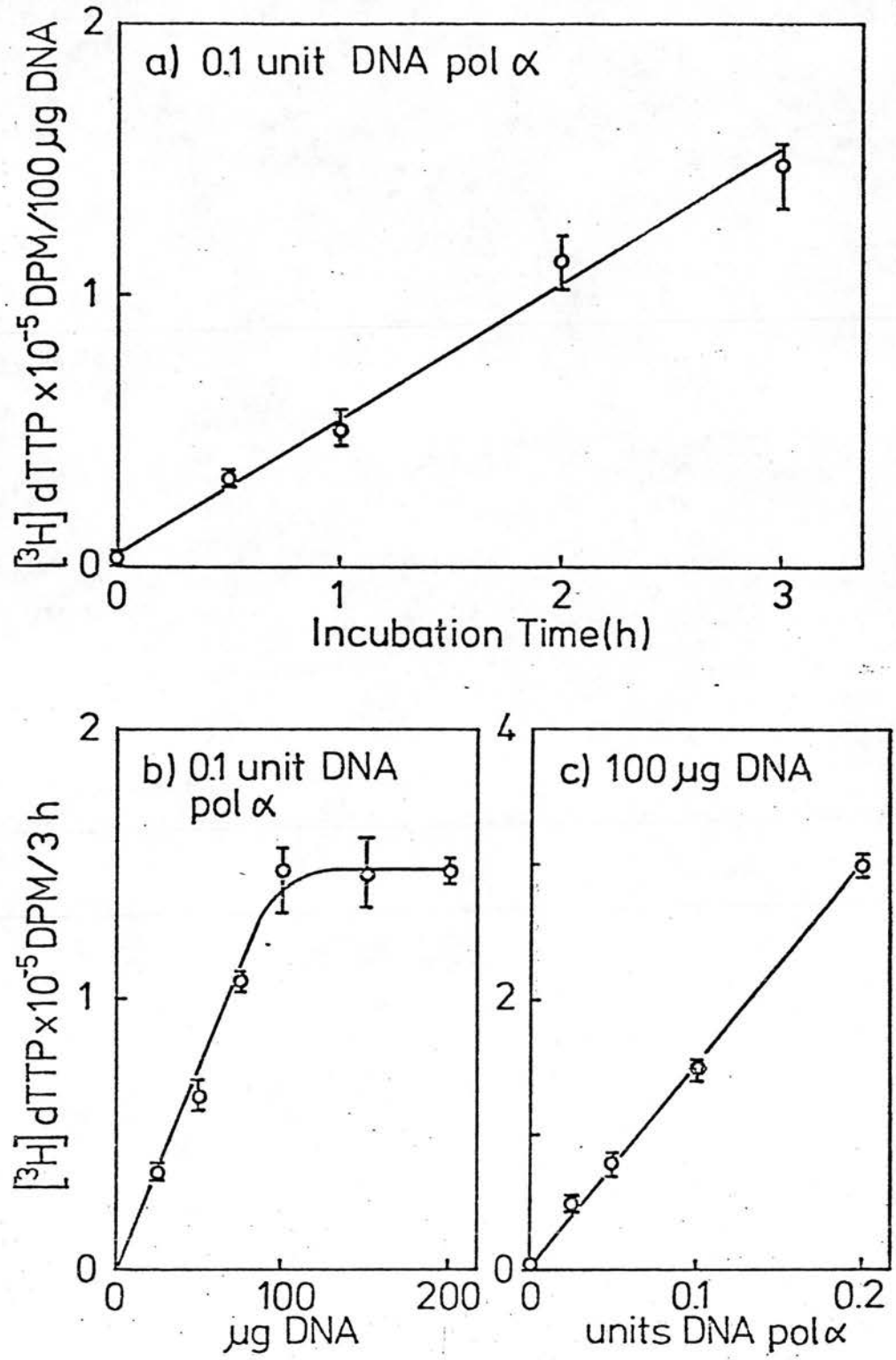
Figure 2.


300
200
100
0
0
1.0
2.0
3.0
[3H] dTTP Incorporation in DNA
(% Control Activity)
Sulfate / Hexosamine (Molar Ratio)


[3H]HEPARIN ×10-5DPM/200µg DNA
0
1
2
3
INCUBATION TIME (h)
[3H]HP ×10-5DPM/3 h
0
1
2
3
0
100
200
300
µg DNA


a) 3 µg Histone
Relative Light Scattering
200
100
0
0
10
20
µg GAG
b) 5 µg Glycosaminoglycans
Relative Light Scattering
200
100
0
0
5
10
µg Histone

RELATIVE LIGHT SCATTERING
200
100
Histones (unfractionated)
Hep
C6S
C4S
HS
N-deS Hep
KS
HA
deS Hep
Histone H1
Hep
C4S
HS
C6S
N-deS Hep
KS
deS Hep
HA
Histones H2A+H3
Hep
N-deS Hep
HS
C4S
C6S
KS
HA
deS Hep
Histone H2B
N-deS Hep
HS
C6S
C4S
Hep
HA
KS
deS Hep
Histone H4
Hep
N-deS Hep
C4S
HS
C6S
KS
deS Hep
HA


a) 0.1 unit DNA pol α
[3H] dTTP x10-5 DPM/100 µg DNA
Incubation Time(h)
b) 0.1 unit DNA pol α
[3H] dTTP x10-5 DPM/3 h
µg DNA
c) 100 µg DNA
units DNA pol α

# SELECTIVE RELEASE OF THE DISACCHARIDE 2-ACETAMIDO-2-DEOXY-3-O-(β-D-GALACTOPYRANOSYL)-D-GALACTOSE FROM EPIGLYCANIN BY ENDO N-ACETYL α-D-GALACTOSAMINIDASE

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ABSTRACT

Epiglycanin, the major glycoprotein of TA3-Ha mammary carcinoma ascites cell was radiolabelled with tritium in the terminal galactose and N-acetyl galactosamine residues. Alkaline-borohydride treatment, reported to release five O-glycosyl-linked chain types from epiglycanin [J. Biol. Chem. 254 (1979) 12153], resulted in the cleavage of 98-99% of the radioactivity from the protein. Of this, 63% of the radioactivity from epiglycanin and 70% from asialoepiglycanin co-migrated with an authentic sample of 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-D-galactitol on a column of Bio-Gel P-6. Incubation of [$^{3}$H-galactose]epiglycanin with endo-N-acetyl-α-D-galactosaminidase (D. Pneumoniae), and fractionation of the mixture on a column of Bio-Gel P-4 gave only one oligosaccharide peak containing 62 and 70%, respectively, of the radioactivity of epiglycanin and asialoepiglycanin. This oligosaccharide co-migrated with authentic 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-D-galactose [I] on Bio-Gel P-2 and P-4 columns and on paper chromatograms.

Results of experiments in which unlabelled epiglycanin was treated with enzyme and the products analyzed, by three different methods, suggested that 78-85% of [I] had been cleaved. Another enzyme, N-acetyl-α-D-galactosaminyl oligosaccharidase from C. Perfringens, exhibited similar specificity and cleaved 65% of the radioactivity from [$^{3}$H-galactose]asialoepiglycanin which was eluted from a Bio-Gel P-2 column as the disaccharide [I].

INTRODUCTION

Epiglycanin, glycoprotein of about 500,000 molecular weight, is the major cell-surface component of the mouse TA3-Ha mammary carcinoma ascites cell[1,2]. Immunochemical and chemical evidence has shown that about 60% of the carbohydrate in epiglycanin is present as β-D-Gal(1→3)-D-GalNAc bound by an O-glycosyl linkage to serine and threonine[3]. The anomeric nature of the glycopeptide linkage has not been reported, even though, based on an analogy to other glycoproteins containing similar structures[4-6], an α-anomeric linkage was considered likely. In order to obtain an uneqivocal proof for the configuration of this linkage we examined the effect of endo-N-acetyl-α-D-galactosaminidase on epiglycanin. In addition, we were interested in partial enzymatic deglycosylaton of epiglycanin in order to investigate the specificity of the anti-epiglycanin antibody[7]. Our results show that the disaccharide β-D-Gal(1→3)-D-GalNAc is linked to serine or threonine via an α-anomeric linkage, and that the disaccharide can be virtually quantitatively removed from intact epiglycanin by the enzyme.

EXPERIMENTAL

Epiglycanin was purified from TA3-Ha ascites cells as described previously[1,3]. Endo-N-acetyl-α-D-galactosaminidase and neuraminidase were purified from culture filtrates of Diplococcus pneumoniae[8,9]. These preparations were free from contaminating glycosidases and proteases[8,9]. Vibrio cholerae neuraminidase was purchased from Calbiochem, La Jolla, CA. Jack bean exo-β-D-galactosidase[10] and Escherichia freundii endo-β-D-galactosidase[11] were gifts from Prof. Y-T. Li, Tulane University, New Orleans, LA. N-acetyl-α-D-galactosaminyl-oligosaccharidase (Clostridium Perfringens) was purchased from Bethesda Research Laboratories, Rockville, MD. Galactose oxidase and horse radish peroxidase were bought from Worthington Biochem. Corp., Freehold, N.J. $NaB^3H_4$ (340mCi/mmol) was purchased from New England Nuclear, Boston, MA.

Asialoepiglycanin was prepared by treatment of epiglycanin with Vibrio cholerae neuraminidase (1 unit) in 200µl 0.1M sodium acetate buffer, pH 5.6 containing 1mM $Ca^{2+}$ at 37° for 24h.

[$^3$H]-Galactose-labelled epiglycanin and asialoepiglycanin were prepared by treatment with galactose oxidase followed by $NaB^3H_4$ according to the method of Morell and Ashwell[12]. After heat inactivation of the enzymes the labelled samples were recovered by exhaustive dialysis against water followed by lyophilization.

Digestion with enzymes: Treatment with endo-N-acetyl-α-D-galactosaminidase from D. Pneumoniae was performed in 50mM Tris/maleate buffer, pH 7.0, with 2mU of enzyme at 37°C for 24h in a volume of 50µl. In some experiments the treatment was continued for 72h, with addition of enzyme (2mU) every 24h. Treatment with E. Freundii endo-β-D-galactosidase was in 0.05M sodium acetate buffer, pH 5.8, at 37°C for 24h[11]. Incubation with C. Perfringens N-acetyl-α-D-galactosaminyl oligosaccharase was in 50mM potassium phosphate buffer, pH 6.5, with 10 mU of enzyme at 37°C for 72h[13]. All digestions were terminated by heating at 100°C for 2-5 min.

Gel filtration: Columns of Bio-Gel P-2 (minus 400 mesh), P-4, P-6 (200-400 mesh) and P-100 (100-200 mesh) were equilibrated and eluted with 0.1M pyridine/acetic acid, pH 5.0.

RESULTS AND DISCUSSION

Characterization of [$^3$H]-galactose-labelled epiglycanin. The specific activities of [$^3$H]-epiglycanin and [$^3$H]-asialoepiglycanin prepared by treatment with galactose oxidase followed by sodium borotritide were 13 x $10^4$ and 20 x $10^4$ DPM per µg, respectively. On acid hydrolysis of the labelled asialoepiglycanin (1N HCl, 100°, 5h) and examination of the products by paper chromatography using 1-butanol/pyridine/water (6:4:3 v/v) as solvent it was found that 91% of the tritium was associated with galactose and 9% with galactosamine. Previous

results have shown that about 19% of the N-acetylgalactosamine residues in epiglycanin are present as single non-reducing units[3]. The lower than expected level of labelling of GalNAc may be due to the unavailability of these residues for the galactose oxidase due to steric reasons[14] and to the observation that non-reducing terminals of GalNAc is a poorer substrate than Gal for the enzyme (Bhavanandan, unpublished results). Bonnet monkey cervical mucin[15] labelled similarly yielded on hydrolysis [$^3$H]-galactose (64%) and [$^3$H]-galactosamine (36%).

On alkaline borohydride treatment[16] of the [$^3$H]-labelled epiglycanin and asialoepiglycanin, followed by chromatography on a calibrated column of Bio-Gel P-6, it was found that 98 and 99%, respectively, of the labelled material was released by β-elimination. Sixty-three and seventy percent, respectively, of the eliminated oligosaccharides co-migrated with authentic 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-D-galactitol isolated from fetuin[5,17].

Action of endo N-acetyl-α-D-galactosaminidase. The result of a typical experiment in which an epiglycanin sample was treated with the endo-N-acetyl-α-D-galactosaminidase and the digest examined by gel filtration is illustrated in Figure 1. After 24h incubation, between 43 to 50% of the label was released as a disaccharide which co-migrated with authentic 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-D-galactose on Bio-Gel P-4 (Figure 1) and P-2 columns (not illustrated). Incubation for longer periods (up to 72h) or isolation of the material excluded from the Bio-Gel P-2 or P-4 column (see Figure 1) and retreatment with the enzyme resulted in release of additional quantities of the disaccharide. A maximum of 62% and 70% of the label in epiglycanin and asialoepiglycanin, respectively, could be released as the disaccharide.

The product released from [$^3$H]-asialoepiglycanin was recovered by combining fractions 78-86 (Figure 1) and lyophilizing to remove pyridine/acetic acid. On paper chromatography the recovered material comigrated with β-D-[$^3$H]-Gal(1→3)

D-GalNAc isolated from asialoglycophorin A and with β-D-Gal(1→3)-D GalNAc isolated from fetuin (Figure 2). Another portion of the material was dissolved in 50μl of 0.05M citrate/phosphate buffer, pH 4.0, and incubated with 2 units of jack bean β-galactosidase at 37° for 29h. Carrier galactose (1mg) was added to the digest and the mixture chromatographed on a Bio-Gel P-2 column. Thirty-seven percent of the radioactivity was coeluted with galactose (Figure 3), confirming that the disaccharide consists of terminal [$^3$H]-Galactose linked in a β-configuration[18]. The partial cleavage of the disaccharide is due to the fact that this enzyme is known to act very poorly on galactose linked β1 3 to the aglycone[10]. In fact, under the same incubation conditions as above this enzyme failed to release [$^3$H]-galactose from [$^3$H]-asialoepiglycanin or from the reduced disaccharide, β-D-[$^3$H]Gal(1→3)GalNAcOl, isolated from asialoepiglycanin by alkaline borohydride treatment. Thus, it appears that the nature of the aglycone influences the activity of jack bean β-galactosidase on galactose in β 1→3 linkages.

To determine on a mass basis the percent of disaccharide, 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-D-galactose, released from epiglycanin we carried out the following experiments. Epiglycanin was incubated with endo N-acetyl-α-D-galactosaminidase alone or in the presence of D. pneumoniae neuraminidase, at 37° for 48h. The released disaccharide was quantitatively determined by the modified Morgan-Elson assay[19] using GalNAc as standard. In calculating the amount of the disaccharide released, the report that GalNAc substituted at position 3 gives 60% more color than unsubstituted GalNAc[20] was taken into consideration. It was found that 15μg of epiglycanin yielded 17.9 and 21.4 nmol of the disaccharide when treated with the endoenzyme alone or with the endoenzyme and neuraminidase, respectively. This corresponds to the release of 60% and 73%, respectively, of total GalNAc as the disaccharide.

In other experiments, 66μg aliquots of epiglycanin were incubated with

buffer alone or with the endo-enzyme (6mU) and neuraminidase (42mU). The digests were fractionated on pre-calibrated Bio-Gel P-2 columns to yield undigested material and enzyme (void volume peak) and the released disaccharide (included peak). An enzyme control was also similarly fractionated. Each of the fractions was hydrolyzed (6N HCl, 110°, 24h in vacuo) and the products analyzed on a Dionex amino acid analyzer equipped with a fluorescence detector. The results are summarized in Table 1. It is clear that about 63% of the total GalNAc was released from epiglycanin which had been treated with both the endoenzyme and neuraminidase.

In further experiments, 200µg of the glycoprotein was treated with the endoenzyme and neuraminidase and the digest fractionated on a column of BioGel P-100. The undigested material, as well as the released oligosaccharides, were recovered, subjected to methanolysis, and the glycosides analyzed as per-trimethylsilylated methyl glycosides by gas liquid chromatography[21]. The composition of the high-molecular-weight (epiglycanin) peak suggested that 70% of the Gal and 62% of the GalNAc, but no GlcNAc, had been cleaved. These results suggest that as much as 78-85% of the disaccharide had been released from the epiglycanin.

Our results clearly establish that the major carbohydrate component of epiglycanin is β-D-Gal(1→3)-D-GalNAc (which is partially substituted by NeuNAc), and that this disaccharide is linked to the protein via an α-anomeric linkage. Further, we have shown that up to about 80% of the disaccharide may be released from asialoepiglycanin by endo-N-acetyl-α-galactosaminidase.

Action of N-acetyl-α-D galactosaminyl oligosaccharidase. A minor portion of the carbohydrate in epiglycanin is present as a hexasaccharide and as a tetrasaccharide[3]. Since the Clostridium perfringens N-acetyl-α-D-galactosaminyl oligosaccharidase has been reported to cleave oligosaccharides larger than disaccharides which are bound by O-glycosyl linkages to protein[13], we tested

the action of this enzyme on [$^3$H]-asialoepiglycanin. The results obtained on gel filtration of the incubation mixture on a column of Bio-Gel P-4 were identical to those obtained with D. Pneumoniae endo-N-acetyl-α-D-galactosaminidase. About 65% of the cleaved material comigrated with β-D-gal(1→3)GalNAc, and no labelled components corresponding to higher oligosaccharides were detected; the tetrasaccharide β-D-[$^3$H]Gal(1→4)β-D-GlcNAc(1→3)β-Gal(1→3)-β-D-GalNAc was expected. Retreatment of the material eluted at the void volume of the Bio-Gel P-4 column with the same enzyme also failed to release any tetrasaccharide.

Action of E. Freundii endo-β-D-galactosidase. When the excluded asialo-material from the Bio-Gel P-4 column (Figure 1) was treated with E. Freundii endo-β-galactosidase, and the digest chromatographed on a Bio-Gel P-4 column, the results illustrated in Figure 4 were obtained. About 14% of the radioactivity eluted in the same region as maltotriose. Insufficient material prevented further examination of the released saccharide. Tentatively, it appears that this endo-β-galactosidase may have released the trisaccharide, β-D-Gal(1→4)-β-D-GlcNAc(1→3)-β-D-Gal, from the above tetrasaccharide.

The specificity of endo-N-acetyl-α-D-galactosaminidase, as well as N-acetyl-α-D-galactosaminyl oligosaccharidase, for cleaving the disaccharide in epiglycanin suggests a unique role for this carbohydrate chain. Although the disaccharide structure itself has been identified in a variety of mammalian tissues and cells[3], it represents not only the predominant carbohydrate structure in epiglycanin, but, as part of a glycopeptide structure, it serves uniquely as a receptor for either the anti-epiglycanin antibody[7] and the lectin from Vicia graminea beans[22]. Whether or not this chain is involved in the suggested antigen-masking function[23] of epiglycanin has not yet been determined.

ACKNOWLEDGMENTS

This investigation was supported in part by grants from the National Cancer

Institute, National Institutes of Health (CA 15483 and CA 08418). We wish to thank Mrs. Anne Katlic and Mrs. Cyla Silber for excellent technical assistance; Mr. Kenneth B. Miller for performing the amino acid analysis and Mr. Keyes B. Linsley for the carbohydrate analysis. We are also grateful to Dr. Y-T. Li for the providing the exo- and endo-β-galactosidases.

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Table 1: Hexosamine and amino acid composition of epiglycan before and after treatment with D. Pneumoniae endo-α-N-acetyl galactosaminidase and neuraminidase and fractionation on BioGel P-4 column

| Amino Acids | Untreated | Incubated with Buffer | | Incubated with Enzymes | |
|---|---|---|---|---|---|
| | | Excluded material | Included material | Excluded Material | Included material |
| | | nmol. per 33µg Epiglycanin | | | |
| Asparatic Acid | 0.28 | 1.12 | 0 | 0.24* | 0* |
| Threonine | 13.10 | 9.92 | 0.12 | 11.14 | 0.02 |
| Serine | 8.79 | 9.38 | 0 | 8.54 | 0.04 |
| Glutamic Acid | 0.44 | 1.44 | 0 | 0.58 | 0.12 |
| Glycine | 2.33 | 2.56 | 0 | 2.48 | 0 |
| Alanine | 3.38 | 2.40 | 0 | 2.79 | 0 |
| Voline | 0.43 | 0.40 | 0 | 0.31 | 0 |
| Isoleucine | 0.18 | 0.23 | 0 | 0.12 | 0 |
| Leucine | 1.06 | 0.86 | 0 | 0.89 | 0.01 |
| Phenyl Alanine | 0.05 | 0.16 | 0 | 0.02 | 0 |
| Glucosamine | 0.81 | 1.07 | 0.15 | 1.11 | 0.18 |
| Galactosamine | 10.01 | 9.88 | 0.12 | 3.53 | 6.03 |

*The values in these columns are corrected for contributins from the enzymes.

Figure Legends

Fig. 1: Elution profiles on Bio-Gel P-4 of [$^3$H]-epiglycanin (●——●) and [$^3$H]-asialoglycophorin (O----O) after treatment with endo-α-N-acetyl galactosaminidase. The treated samples were mixed with [$^{14}$C]-glucose (▲——▲) and chromatographed on a Bio-Gel P-4 column (0.9 x 108cm). The column was eluted with 0.1M pyridine/acetic acid, 1ml fractions collected and analyzed for radioactivity by liquid scintillation counting. The void volume ($V_o$) and elution position of authentic β-D-Gal(1→3)GalNAc are indicated.

Fig. 2: Paper chromatography of the endo-α-N-acetylgalactosaminidase released products from [$^3$H] asialoepiglycanin (———) and [$^3$H] asialoglycophorin ( ----- ). The [$^3$H]-labelled products recovered from the Bio-Gel P-4 fractions 78-86 (Figure 1) were chromatographed using butylacetate/acetic acid/water (3:2:1, v/v) as solvent. The paper strips containing the labelled samples were cut into 1 cm wide pieces, extracted with 1ml water and the extracts analyzed for radioactivity. The migration positions of standard sugars were detected by the silver nitrate staining procedure.

Fig. 3: Elution profiles on a column of Bio-Gel P-2 (0.9 x 70cm) of β-D-[$^3$H]-Gal(1→3)GalNAc (O-----O) and [$^3$H]-asialoepiglycanin (●——●) after treatment with jack bean β-galactosidase as described in the text. The column was eluted with 0.1M pyridine/acetic acid, 1ml fractions were collected and aliquots analyzed for radioactivity and galactose (by the phenol-sulfuric acid reaction). The elution pattern of [$^{14}$C]-glucose (□——□) and the peak elution positions of blue dextran ($V_o$) and NeuNAc are indicated.

Fig. 4: Elution profile on Bio-Gel P-2 of endo-α-N-acetyl galactosaminidase resistant [$^3$H]-asialoepiglycanin after treatment with E. Freundii endo-β-galactosidase. The enzyme digest was mixed with [$^{14}$C] glucose (Δ-----Δ) and

chromatographed on a Bio-Gel P-2 column (0.9 x 68 cm). The column was eluted with 0.1M pyridine acetic acid, 1ml fractions were collected and analyzed for radioactivity. The peak elution positions of blue dextran ($V_o$), NeuNAc and maltotriose are indicated.

$^3$H DPM x $10^{-2}$
4
8
12
40
60
80
100
FRACTION NUMBER
$V_o$
Gal → GalNAc
$^{14}$C - Glucose

Figure 2

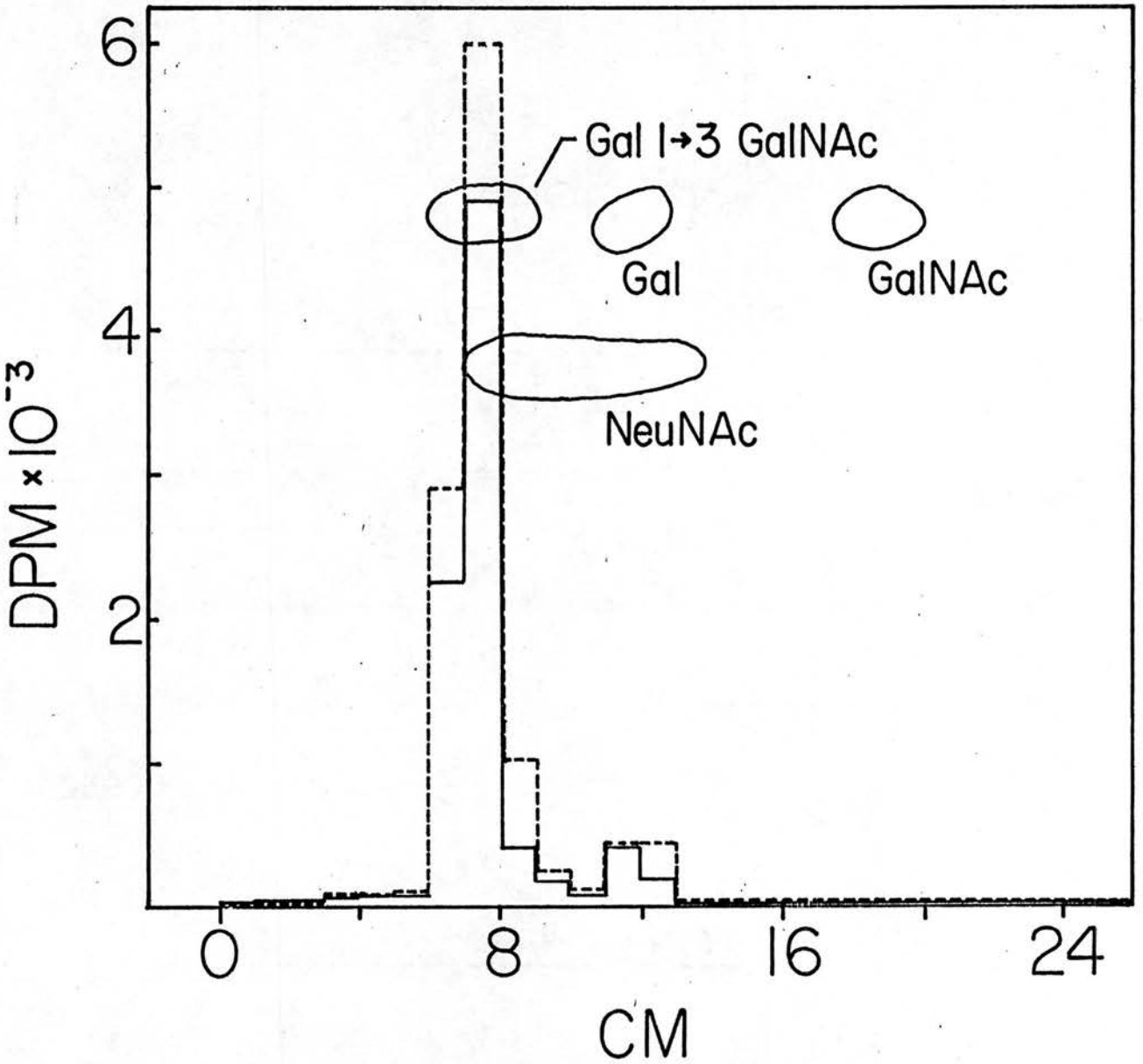

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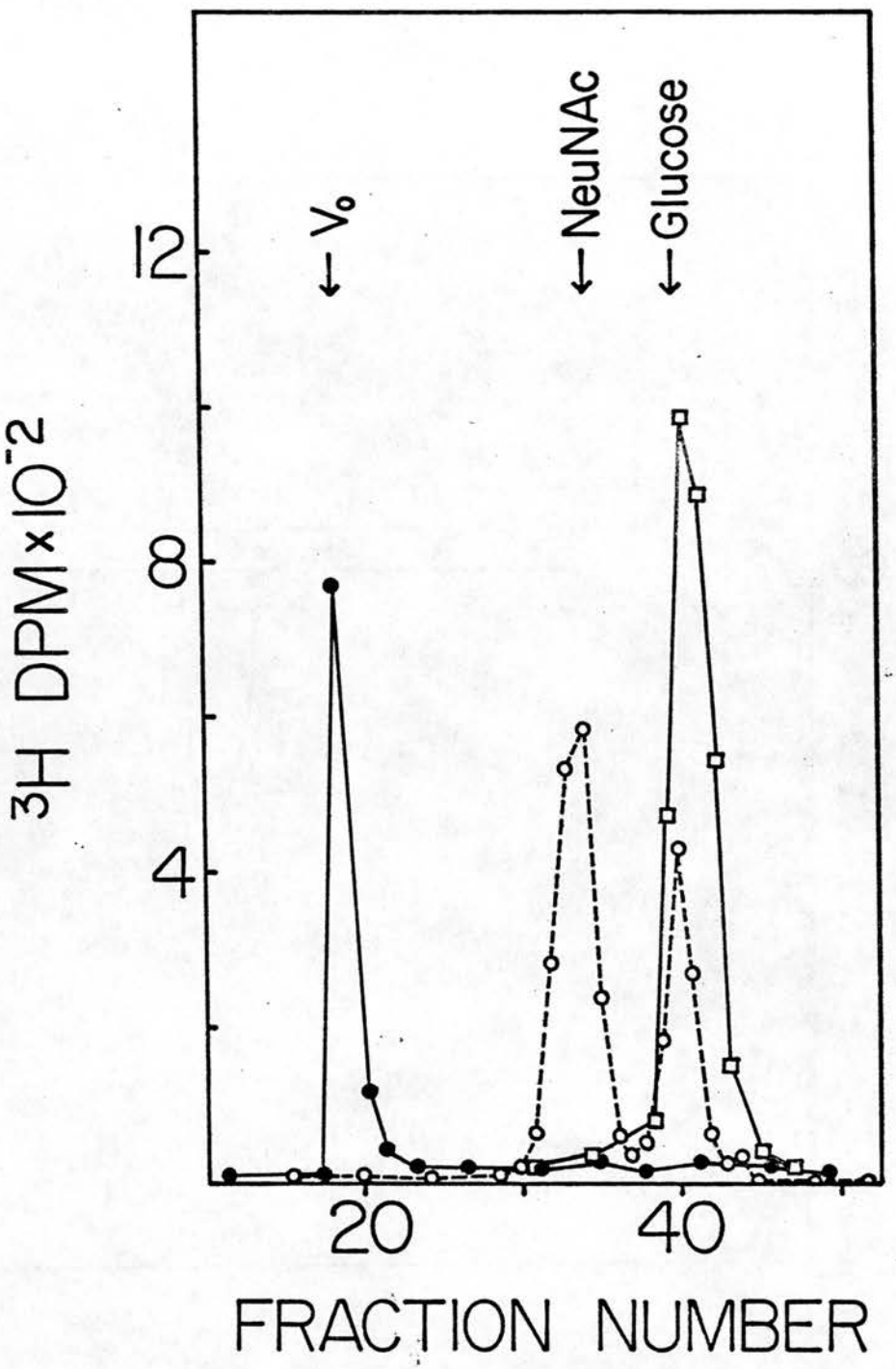
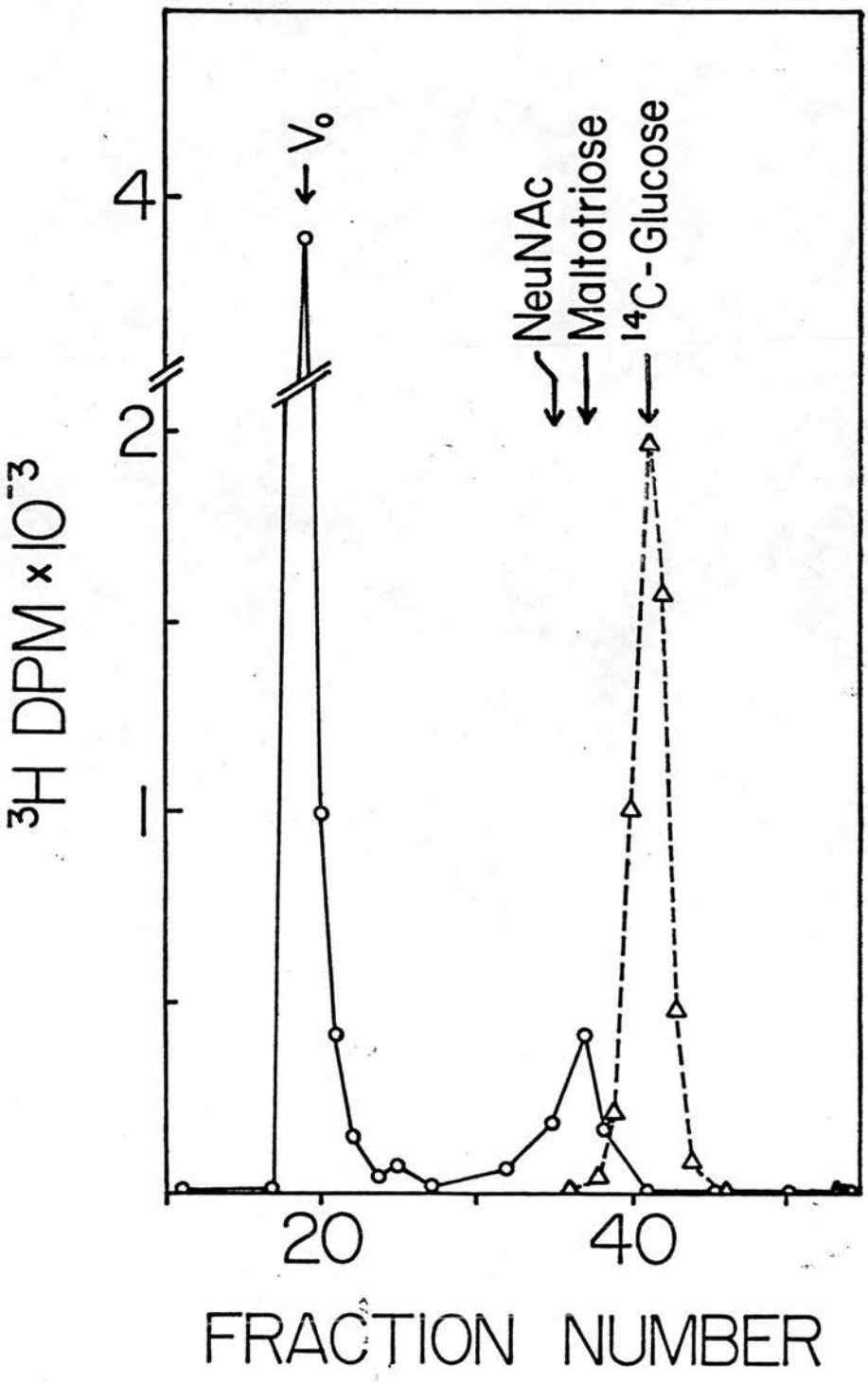
Figure 3



Bhavanandan and Codington

Figure 4



# CHARACTERISTICS OF AN ANTIBODY TO EPIGLYCANIN*+

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Running title: Antibody to Epiglycanin

Footnotes

*This work was presented in part at the VIth International Symposium on Glycoconjugates, Tokyo, Japan, 1981.

+Supported by USPHS, NIH grants CA-08418, CA-18600, CA-17686; and grants from the National Arthritis Foundation, the L. H. Bendit Foundation and the HOR Foundation.

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Abstract

An anti-epiglycanin antibody was induced by repeated inoculation of either rabbits or mice with viable TA3-Ha mammary carcinoma ascites cells. Picogram quantities of epiglycanin were detected in a radioimmunoassay by inhibition of the formation of the complex of the antibody and $^{125}$I-epiglycanin. The probable involvement of carbohydrate in the antigenic determinant for the antibody was shown by the loss of inhibitory activity following periodate oxidation. The requirement of the disaccharide, O-β-D-galactopyranosyl-(1→3)-N-acetyl-α-D-galactopyranose, attached to L-serine or L-threonine, was demonstrated by the action of endo-N-acetyl-α-D-galactosaminidase (D. pneumoniae), which cleaved this type of chain (75-80% in epiglycanin) only, with 99% loss of activity. The resulting partially deglycosylated epiglycanin was inactive, as was the isolated disaccharide chain, suggesting the requirement for specific peptide linkages. This result is consistent with the inactivity or the weak activity exhibited by glycoproteins with similar disaccharide chains but with different amino acid sequences. Epiglycanin was at least 500-fold more active than any other substance tested. Both chromatographic and immunologic data suggest that the rabbit antibody was of the IgG and IgM classes.

# INTRODUCTION

Long rod-like molecules of epiglycanin dominate the surfaces of the TA3-Ha and TA3-MM/1 ascites cells and appear to mask histocompatibility antigens. This phenomenon may explain, at least in part, the allotransplantability of these cells. The characteristics of the TA3 tumor and of epiglycanin have been reviewed (Codington & Frim, 1982). Previous attempts to detect anti-epiglycanin antibody in the serum of either rabbits or mice injected with the glycoprotein emulsified in Freund's complete adjuvant were unsuccessful. We report herein the presence of anti-epiglycanin antibody in high titre in the sera of both mice and rabbits inoculated with a suspension of viable TA3-Ha ascites cells emulsified in Freund's complete adjuvant followed by repeated intravenous injections of the tumor cells. We describe the characteristics of these antibodies and the development of a radioimmunoassay for the detection of epiglycanin based on these antibodies.

## MATERIALS AND METHODS

### Cells

TA3-Ha and TA3-St ascites cells of the strain A mouse were obtained as described by Codington et al., (1979). The description of these cells was reported by Hauschka et al., (1971) and Cooper et al., (1979). Tumor cells were maintained by passage in A/HeJ mice (Jackson Laboratories, Bar Harbor, ME 04609), and were harvested on day 7 following the intraperitoneal inoculation of $10^5$ TA3-Ha or $10^6$ TA3-St cells. Cells were washed several times with phosphate buffered saline (PBS) (Dulbecco & Vogt, 1954) and their concentration was determined.

### Antigens

Epiglycanin of 500,000 molecular weight (Fraction II) was isolated from the ascites fluid of A/HeJ mice bearing TA3-Ha ascites cells (Codington et al., 1979). Fragments of epiglycanin (avg. molecular weight 200,000, Fraction A) were obtained by incubation of viable TA3-Ha ascites cells with TPCK-trypsin and were purified by gel filtration (Codington et al., 1979). Fraction C was obtained by further purification of Fraction A (Slayter & Codington, 1973). Reduced oligosaccharides of one, two, three and four carbohydrate residues were obtained by alkaline borohydride reduction of Fraction A,

followed by gel filtrate ion chromatography (van den Eijnden et al., 1979). Glycopeptide BH (borohydride) was obtained by the same procedure, and its effluent volume suggested a molecular weight of about 4,000.

3-O-β-D-galactopyranosyl-(1→3)-O-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-(1→3)-N-p-toluenesulfonyl-L-serine was supplied by Professor T. Osawa of Tokyo University, and O-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranosyl)-(1→3)-L-threonine by Professor H. Paulsen of the University of Hamburg. Glycophorin A (Tomita et al., 1978) was a gift from Dr. H. Furthmayr, Department of Pathology, Yale University. The freezing-point-lowering glycoprotein from Antarctic fish (Osuga & Feeney, 1980) was a gift from Dr. R. E. Feeney, University of California, Davis, and fetuin (Spiro & Bhoyroo, 1974) was a gift from Drs. Robert and Mary Jane Spiro, Department of Biological Chemistry, Harvard Medical School. The bovine lubricating glycoprotein (Garg et al., 1980) was obtained as a gift from Drs. David Swann and Hari Garg of the Massachusetts General Hospital. 2-Acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-D-glucose (N-acetyllactosamine) and methyl 2-acetamido-2-deoxy-β-D-galactopyranoside were purchased from Pfanstiehl Laboratories, Waukegan, IL.

Enzymes

Neuraminidase (3.2.1.18) from Vibrio cholerae (1 U/ml) was obtained from Calbiochem-Behring, San Diego, CA. Endo-N-acetyl-α-D-galactosaminidase was prepared from D. pneumoniae, as described (Bhavanandan et al., 1976; Umemoto et al., 1977). No protease was detected when the enzyme was tested with either azocasein or methemoglobin as substrate. β-D-Galactosidase (3.2.1.23, Canavalia ensiformis, 23 U/ml) was a gift from Dr. Y.-T. Li, Tulane Medical Center, New Orleans, LA. α-D-Mannosidase (3.2.1.24, C. ensiformis, 10 U/mg) was obtained from Boehringer Mannheim, Indianapolis, IN, and N-acetyl-α-D-galactosaminidase (3.2.1.49, C. lampas, 0.4 U/mg) was purchased from Miles Laboratory, Kankakee, IL. N-Acetyl-α-D-galactosaminidase (3.2.1.49) from C. perfringens was purchased from Bethesda Research Laboratories, Rockville, MD. β-D-Hexosaminidase (3.2.1.52) was obtained from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. N-Acetyl-α-D-galactosaminidase (3.2.1.49) from pig liver was donated by Dr. B. Weissmann, University of Chicago. L-1-Tosylamido-2-phenylethyl-chloromethylketone trypsin (3.4.4.4, TPCK-trypsin) and galactose oxidase (1.1.3.9, 67 U/mg) were purchased from Worthington Biochèmical Corp., Freehold, NJ).

Reagents

N-Succinimidyl-3-(4-hydroxy-5-$^{125}$I-iodophenyl) propionate (Bolton Hunter Reagent) (Bolton & Hunter, 1973), (2000 Ci/mmol) was purchased from New England Nuclear Corp., Boston, MA, and used within one week.

$^{125}$I-Epiglycanin

A stream of dry air was passed into a vial containing 1.0 mCi of the Bolton-Hunter Reagent (2000 Ci/mmol), as received from the supplier, to remove the benzene solvent. To the residue was added a solution of 15 µg of epiglycanin (500,000 molecular weight) in 30 µl of 0.1 M borate buffer (pH 8.5). After 19 hours at 0-4°, $^{125}$I-epiglycanin was separated from unreacted reagent and reaction by-products by elution with PBS from a column (0.6 x 21 cm) of Bio-Gel P-4 (200-400 mesh). The solution of radiolabelled epiglycanin was stored at -20°.

Induction of anti-epiglycanin antibody

A. In mice

An emulsion (0.1 ml) composed of equal volumes of PBS containing 1 x $10^6$ TA3-Ha ascites cells per ml and Freund's complete adjuvant was injected into an axilla of each of four male C57BL mice 7 weeks of age. Injections of $10^5$ cells in 0.1 ml of PBS were given in the tail vein of each mouse

each week for 8 weeks. As a control, one mouse was given an initial injection of Freund's complete adjuvant with PBS, and was given additional injections of 0.1 ml of PBS, without the tumor cells, according to the same schedule. Mice were bled from the retroorbital venous plexus every two weeks, beginning at week 1 and continuing until week 9. The serum samples were stored at -20° until tested in the radioimmunoassay for antibody activity.

B. In rabbits

Each of two New Zealand white rabbits was inoculated in the four foot pads with a total of 4 ml of an emulsion prepared from 2.0 ml of Freund's adjuvant and 2.0 ml of PBS containing $1.2 \times 10^7$ TA3-Ha ascites cells. Thereafter, each rabbit was injected intravenously, usually at two or three week intervals, with $2 \times 10^6$ TA3-Ha cells in 2.0 ml of PBS. The rabbits were bled before each injection. Nineteen months following the initial injection of TA3-Ha ascites cells, 120 µg of epiglycanin in Freund's complete adjuvant was injected intramuscularly. Serum was obtained after 6 weeks and after an additional 4 weeks; during these intervals 4 and 3 intravenous injections, respectively, of $2 \times 10^6$ TA3-Ha ascites cells, were given. All radioimmunoassays reported herein utilized serum obtained from these latter two bleedings.

Radioimmunoassays

Radioimmunoassays were employed in the determination of anti-epiglycanin antibody activity and in the determination of the concentration of epiglycanin or epiglycanin-like material in solution. For determining the antibody titre, a mixture of 100 µl of rabbit or mouse antiserum at appropriate 2-fold dilutions and 50 µl of $^{125}$I-epiglycanin (3 ng, 1600-2200 cpm) was incubated for 16-20 hours at 4° in 1.2 ml plastic centrifuge tubes. Precipitation was completed by the addition of a second antigen-antibody system consisting, for the rabbit antibody, of 0.20 ml of normal rabbit serum (1:50) and 0.15 ml of goat anti-rabbit IgG antiserum, and for the mouse antibody, 0.20 ml of normal mouse serum (1:50) and 0.075 ml of rabbit anti-mouse antiserum (Cappel Laboratories Inc., Cochranville, PA). After 16-20 hours at 4°, 0.30 ml of PBS was added. After centrifugation, the radioactivity of each supernatant and precipitate was determined by crystal scintillation spectrometry.

Inhibitory activity was determined by adding the inhibitor in 50 µl of PBS to 100 µl of rabbit or mouse anti-epiglycanin antiserum at about 1:1000 or 1:200 dilutions, respectively, in a 1.2 ml tube and incubating the mixture at 37° for 60 min and at 22° for 60 min. Thereafter, $^{125}$I-epiglycanin (50 µl, 3 ng, 1600-2200 cpm) was added to each tube, and the mixture

incubated at 4° for 16-20 hours. The second antigen-antibody mixture, as described above, was then added, and incubation continued for 16-20 hours at 4°. The radioactivity in both the supernatants and precipitates was determined after centrifugation. The proportion of radioactivity in the precipitates was plotted versus the concentration of antigenic substances added. Activity was determined by comparison with a standard curve prepared with epiglycanin as the inhibitor.

Characterization of antibody induced in the rabbit

(A) Fractionations

Antiserum of maximum titre (1.0 ml) was applied to a column (1.0 x 15 cm) of DEAE-cellulose (DE-52, Whatman Chemical Separation, Inc., Clifton, NJ) and fractions were eluted, successively, with 0.01 M sodium phosphate (pH 7.5), 0.02 M sodium phosphate (pH 6.2), and 1.0 M sodium chloride, according to a modification of the procedure of Fahey (1967). Fractions (1.5 ml) were tested for protein content (Lowry et al., 1951) and for the ability to bind $^{125}$I-labelled epiglycanin in the radioimmunoassay.

For the comparison of the elution characteristics of the antibody in gel filtration with those of known antibody classes (Fireman et al., 1963), fractions from the DE-52 column constituting peaks of antibody activity, after dialysis against 0.1 M pyridine acetate and lyophilization, were dissolved in 0.2 M NaCl and applied to the same column (1.0 x 111 cm) of Sephadex G-200 (Pharmacia, Piscataway, NJ 08854). Fractions (1.5 ml) were eluted with 0.2 M NaCl and tested for protein content and capacity to bind labelled epiglycanin, as described above for fractions from the ion-exchange column.

B. Immunoprecipitation of antibody

Four tubes, each containing anti-epiglycanin antiserum (100 µl) at a dilution of 1:150, were each incubated with 100 µl of goat anti-rabbit IgA, IgG, IgM antiserums (all heavy chain specific; Cappel Laboratories) or buffer at 37° for 60 min and 4° for 20 hours. After centrifugation, 185 µl of supernatant was removed from each tube, and the residues were discarded. The radioimmunoassay was performed to determine the concentration of antibody remaining in the supernatants.

Modification of epiglycanin

For the complete removal of sialic acid, epiglycanin (100 µg) was incubated with 20 µl (0.02 U) of neuraminidase in 500 µl of PBS at 22° for 16 hours. The cleavage of β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactose from epiglycanin was performed, as reported elsewhere (Bhavanandan & Codington, 1982); 200 µg of the glycoprotein (molecular weight 500,000) in 90 µl of 50 mM tris-maleate buffer (pH 6.8) was treated at 0, 24 and 48 hours with 10 µl of the same buffer containing 0.002 U of endo-N-acetyl-α-D-galactosaminidase. The solution was incubated at 37°, and the reaction terminated at 72 hours by freezing. For the reaction with asialoepiglycanin, 0.014 U of neuraminidase (D. pneumoniae) was added to a similar reaction mixture containing endo-N-acetyl-α-D-galactosaminidase at 0 hours.

Periodate oxidation was performed with 30 µg of sodium metaperiodate in a solution of 17 µg of epiglycanin (500,000 molecular weight) in 200 µl of PBS at 22°. Aliquots were removed at appropriate intervals. The reaction was terminated at 20 hours by the addition of 50 µl of ethylene glycol.

[$^{3}$H-Galactose]-epiglycanin

Epiglycanin was radiolabelled in the galactose residues by a modification of the method of Morell and Ashwell (1972). The glycoprotein (100-200 µg) in 0.1 M phosphate buffer (pH 7.0) was incubated with galactose oxidase (5 U, Worthington Biochemical Corp., 150 U/mg) at 37° for 30 min. Sodium borotritide (0.5 mCi, New England Nuclear Corp., 246 Ci/mol) was added, and the mixture incubated at 23° for 2 hours. After the addition of sodium borohydride (0.7 mg) and reaction for 60 min, the mixture was acidified, and borate was removed by repeated evaporation with dry methanol.

## RESULTS

### Induction of antibody

#### A. In the mouse

Epiglycanin-binding activity in the sera of C57BL mice is illustrated in Figure 1A. The level of binding activity had risen markedly 3 weeks after the initial injection of tumor (TA3-Ha) cells and had reached a plateau at about week 4. With continued biweekly injections of the viable cells, this level was maintained for at least 4 additional weeks. The binding of $^{125}$I-epiglycanin by the antiserum at several dilutions, as determined in the radioimmunoassay, is illustrated in Figure 2. In this experiment, approximately 2 ng of the labelled glycoprotein (specific activity, $8 \times 10^5$ cpm/μg) was added to each tube; there was a progressive loss of binding activity on dilution of the antiserum.

#### B. In the rabbit

The appearance of epiglycanin-binding activity in the rabbit is illustrated in Figure 1B. The initial injection of tumor cells, emulsified with Freund's complete adjuvant, was made in the foot pads of two rabbits, as compared with the axillae in the mouse, but periodic intravenous injections with viable cells were performed in each species. Since one of the rabbits died before the completion of the injection schedule, the results are presented only for the surviving

rabbit. The partial results suggest, however, that the epiglycanin-binding capacity in the sera of the two rabbits was approximately the same. Epiglycanin-binding activity was first detected in the serum 4 weeks following the initial injection (Figure 1B). Similar and essentially complete precipitation of $^{125}$I-epiglycanin was observed at dilutions of 1:100 and 1:400 for all of the antisera tested (Figure 1B). Therefore, comparisons among antisera were at a higher dilution (1:1600). The highest binding activity was obtained 7 weeks following the initial injection. Thereafter the binding activity at this dilution decreased; no further effort was made to obtain antisera following this period. More than a year later, however, it was found that the serum of the rabbit still possessed the anti-epiglycanin antibody, and a renewed effort was made to prepare antisera of high antibody content. Following an intensive program of injecting viable cells, two separate bleedings made at a four week interval yielded antisera with high binding activity (Figure 1B). The almost identical binding of $^{125}$I-epiglycanin by various dilutions of the latter two antisera is shown in Figure 3. These two samples of antiserum were used in all subsequent experiments described in this report.

Inhibitory activity

The activity of epiglycanin, isolated from the ascites fluid (Fraction II), in inhibiting the binding of $^{125}$I-epiglycanin to mouse or rabbit anti-epiglycanin antibody is shown in Figure 4. The two experiments were performed with different preparations of labelled glycoprotein; therefore, a direct comparison of the two binding curves is not possible.

The inhibitory activities of a number of glycoproteins and asialoglycoproteins were compared with those of two epiglycanin and asialoepiglycanin fractions (Table I). The results indicate a marked degree of specificity of the antibody for structures present in epiglycanin, since no other substance tested approaches epiglycanin in inhibitory activity. Of the most active glycoproteins tested were those isolated from the cell surfaces of rat mammary carcinoma ascites cells by Carraway et al. (1978). After removal of sialic acid residues, several glycoproteins, such as fetuin, glycophorin and the bovine lubricating glycoprotein, exhibited weak activity.

Antigenic determinant

A summary of the inhibitory activities in the radioimmunoassay remaining after treatment of epiglycanin with various glycosyl hydrolases and with periodate is presented in Table II.

Periodate oxidation destroyed all inhibitory activity, which suggested that carbohydrate linkages were involved in the antigenic determinant. One glycosylhydrolase, endo-N-acetyl-α-D-galactosaminidase, was found to eliminate 99% of the inhibitory activity. It has been reported that this enzyme is capable of cleaving the disaccharide, 2-acetamido-2-deoxy-3-O-β-D-galactopyranosyl-α-D-galactopyranose, linked to serine or threonine in a glycopeptide structure (Figure 5,II) (Umemoto et al., 1978). Of the seven other glycosylhydrolases used, only one, neuraminidase from Vibrio cholerae, displayed a significant effect upon activity, which was increased from 2- to 5-fold. This result is consistent with the finding (van den Eijnden et al., 1979) that treatment of epiglycanin with neuraminidase results in the exposure of approximately 20% more disaccharide chains.

Following the incubation of asialoepiglycanin with endo-N-acetyl-α-D-galactosaminidase, the reaction mixture, which possessed about 1% of the activity of the original asialoepiglycanin sample, was eluted from a column of Bio-Gel P-100, and each fraction was tested for inhibitory activity in the radioimmunoassay (Fig. 6). Only one peak of activity was observed. The active material was eluted in an effluent volumne only slightly greater than that required to elute an unreacted sample of asialoepiglycanin (position a), a result consistent with a 20-25% loss in molecular weight, due to

deglycosylation. No activity was detected at the effluent volume required to elute an authentic sample of the disaccharide (location b, Fig. 6). The carbohydrate analysis of the active material eluted from the column showed proportions of both galactose and N-acetylgalactosamine lower than those of the original epiglycanin sample. On the assumption that no loss in the proportion of N-acetylglucosamine occurred, the enzyme-treated product exhibited a loss of 71% galactose and 62% N-acetylgalactosamine. No loss in mannose was observed. Based upon published values for the proportion of O-glycosyl linked chains in epiglycanin (van den Eijnden et al., 1979), these values suggest that 75-85% of the disaccharide chains were cleaved by the enzyme.

The inhibitory activities of O-linked oligosaccharides found in epiglycanin, or closely related to those present in epiglycanin, are given in Table III. Weak activity (approximately 0.005% of that of epiglycanin) was found in the reduced disaccharide and the reduced sialylated disaccharide. No activity was detected for the disaccharide itself, but this substance was not tested at high concentration. Neither the disaccharide bound by an α-linkage to serine (with a p-toluenesulfonyl residue linked to the amino group of the serine), nor the disaccharide bound to threonine, exhibited any detectable

activity. No activity was detected for the reduced tetrasaccharide containing one N-acetyl-D-glucosamine residue and two D-galactose residues, which had also been isolated from epiglycanin following alkaline borohydride reduction (van den Eijnden et al., 1979). One of the most active inhibitors listed is the glycopeptide BH (borohydride). This material, which resulted from the same reduction with sodium borohydride, was isolated in small yield by gel filtration, and the effluent volume suggests an apparent molecular weight of about 4,000. The analysis by gas-liquid chromatography indicated the presence of both carbohydrate and amino acid residues and was consistent with the presence of several disaccharide (Fig. 5,II) and sialylated disaccharide chains.

Class of antibody

A. Fractionations

Fractionation of anti-epiglycanin antiserum from the rabbit on a column of DEAE cellulose yielded four major protein peaks. The first peak (I) was eluted with 0.01 M phosphate buffer. Antibody activity was present in the fractions constituting this peak. Elution with 0.02 M phosphate buffer (pH 6.2) gave rise to two protein peaks, (II) and (III). Peak (II) exhibited weak antibody activity; peak

(III) possessed no detectable activity. Elution with 1.0 M NaCl produced a large protein peak (IV), which possessed antibody activity. Based upon earlier reports (Fahey, 1967), it was expected that IgG class antibodies would be found in peak (I), and that IgM class antibodies would be present in peak (IV).

The relative apparent molecular weight of antibodies present in peak (I) was compared with those in peak (IV) by elution from the same Sephadex G-200 column. Antibody activity in peak (I) was eluted in the 40-60-ml fraction of effluent (Figure 7A), and that in peak (IV) was eluted in the 31-33 ml fraction (Figure 7B). These elution volumes are consistent with the behavior on a Sephadex G-200 gel filtration column of IgG and IgM molecules, respectively (Fireman et al., 1963).

B. Immunoprecipitation of antibody

Incubation of rabbit anti-epiglycanin antiserum with either goat anti-rabbit-IgG ($\gamma$-chain) anti-rabbit IgM ($\mu$-chain) antiserums resulted in the loss of 54-57% of the antigen-binding activity (Table IV). Incubation with goat anti-IgA ($\alpha$-chain) antiserum resulted in only a 16-18% loss of activity. Based upon the results of this experiment, as well as those of the fractionation experiments, it appears that anti-epiglycanin antibody activity is distributed approximately equally between the IgG and IgM classes of rabbit antibodies.

## DISCUSSION

The surfaces of mammalian cells are generally composed of many different types of macromolecules with potential antigenic activity. It was thus fortuitous that injection of mice or rabbits with TA3-Ha mouse mammary carcinoma ascites cells emulsified in Freund's complete adjuvant initiated a strong immune response to at least one of the glycoproteins, epiglycanin, of the tumor cell surface. This success may be due to the dominant position occupied by epiglycanin at the surface of the TA3-Ha cell. Epiglycanin is not only the most abundant glycoprotein at the surface of the TA3-Ha cell, but probably possesses the greatest molecular weight and molecular length (Codington et al., 1979), factors which may enhance its immunogenic activity in intact cells.

The evidence (Tables II and III) suggests that both the carbohydrate and protein moieties of epiglycanin are required for binding the antibody. The involvement of carbohydrate in the antigenic determinant is strongly supported by the observations (Table II) that both periodate oxidation and incubation with endo-N-acetyl-α-D-galactosaminidase (D. pneumoniae) destroy the binding activity of epiglycanin. This enzyme was shown to be free of proteases (Umemoto et al., 1978).

In a study of its action on epiglycanin (Bhavanandan & Codington, 1982) evidence was obtained that only one type of carbohydrate chain, the disaccharide, 2-acetamido-2-deoxy-3-O-β-D-galactopyranose (Fig. 5,I) was cleaved by the enzyme. Approximately 80% of the total number of chains of this type (van den Eijnden et al., 1979) were cleaved in the incubations. A similar specificity had been demonstrated for synthetic substrates (Umemoto et al., 1978). The observation that none of the other glycosyl hydrolases used in these experiments reduced the binding activity (Table II) was consistent with the involvement of the disaccharide (Fig. 5,II) since none of these enzymes had been found to be capable of cleaving the galactose-N-acetylgalactosamine linkage in the disaccharide (van den Eijnden et al., 1979). The enhancement of binding of epiglycanin to the anti-epiglycanin antibody after neuraminidase treatment may be due to the exposure by this treatemnt of about 20% more disaccharide chains in the molecule (van den Eijnden et al., 1979).

Since the structures of both O-glycosyl (van den Eijnden et al., 1979) and N-glycosyl (Frim et al., in preparation) chains in epiglycanin have been established, it was possible to anticipate the probable effect of each of the enzymes (Table II) on the carbohydrate structure. Thus, the β-D

galactosidase used was incapable of cleaving the β-D-galactopyranosyl moiety in the disaccharide (Fig. 5) but was able to remove the β-D-galactopyranosyl group from a lactosamine-type structure present in tetrasaccharide or desialyated hexasaccharide chains (van den Eijnden et al., 1979), or from a similar structure in an N-glycosyl-linked chain (Li, 1979), if this were present. The failure of the α-D-mannosidase, the β-D-galactosidase and the β-D-hexosaminidase to reduce inhibitory activity strongly supports the conclusion that N-glycosyl-linked chains are not involved in the antigenic determinant.

Since the carbohydrate structure (Fig. 5,I) probably involved in the binding of the antibody is commonplace in mammalian glycoproteins (van den Eijnden, 1979), at least two additional factors may play a role in the antigen-antibody interaction, namely, an unusual amino acid sequence at the site of the carbohydrate chain attachment and a relatively easy accessibility of the binding site to the antibody. Two types of evidence suggest strongly that a peptide moiety is a requirement for binding activity. From Table III it can be noted that little or no inhibitory activity was found for the isolated disaccharide (Fig. 5,I) the reduced disaccharide, or the disaccharide attached to a single amino acid (serine or threonine). Furthermore, the so-called antifreeze glycoprotein (Osuga & Feeney, 1978)

was found to be without activity, even though this material consists of repeated disaccharide units, each attached to the threonine residue of a repeating tripeptide unit. None of the glycoproteins listed in Table I exhibited an inhibitory activity comparable to that of epiglycanin. The most active of these, the asialo-bovine lubricating glycoprotein (Garg et al., 1980) was reported to possess a high concentration of disaccharide (Fig. 5,II) chains. Other active glycoproteins, such as asialoglycophorin (Tomita et al., 1978), asialofetuin (Spiro & Bhoyroo, 1974), and the glycoproteins from rat mammary carcinoma ascites cells (Carraway et al., 1980), were each reported to contain the same disaccharide as found in epiglycanin. Since the amino acid sequence of epiglycanin is not yet known, it is not known whether or not regions of amino acid sequence homologies exist in epiglycanin and other molecules which bind the antibody.

It is of interest that a lectin from Vicia graminea beans appears to bind, with a high degree of specificity, a similar or related structure in epiglycanin (Cooper et al., 1974; Codington et al., 1975). This lectin has also been reported to bind to the disaccharide (Fig. 5,II) present in a glycoprotein of the human erythrocyte surface (Uhlenbruck & Dahr, 1971). Furthermore, the binding was reported to be

dependent upon a specific amino acid sequence at the amino terminal of the polypeptide chain (Duk & Lisowska, 1981; Duk et al., 1982). Further experimentation will be required to determine whether or not the antibody and the lectin bind to the same receptor site in epiglycanin.

The same disaccharide as illustrated in Fig. 5,II, at the human erythrocyte surface, following removal of the sialic acid with neuraminidase, is known as the Thomsen-Friedenreich antigen (Vaith & Uhlenbruck, 1978). Little information appears to be available regarding the possible involvement of amino acids in the antigenic determinant for the Thomsen-Friedenreich antigen. In a recent paper (Bray et al., 1981), however, it was reported that a synthetic hapten, 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-3-O-β-D-galactopyranosyl-α-D-galactopyranoside, a derivative of the disaccharide (Fig. 5,I) when coupled to bovine serum albumin, induced antibody in the rabbit which was capable of agglutinating neuraminidase-treated human erythrocytes. The possible relationship between the antibody induced by the synthetic hapten and that induced by epiglycanin may be of significance in view of the reports (Springer et al., 1977; 1979) that the Thomsen-Friedenreich antigen occurs at the cell surfaces in human mammary carcinoma tissue, but not in normal tissue. This finding, however, has been contradicted by others (Newman et al., 1979; Klein et al., 1979).

The presence at the tumor cell surface of high concentrations of endogenous O-glycoproteins of high molecular weight is associated with allotransplantability (Codington et al., 1973; Sanford et al., 1973; Codington, 1980; Codington & Frim, 1982) and metastatic properties (Cooper et al., 1977) in mouse mammary carcinomas, metastatic properties (Carraway et al., 1978) and xenotransplantability (Buck et al., 1979; Sherblom et al., 1980) in rat mammary carcinomas, and metastatic properties in the B 16 mouse melanoma (Bhavanandan & Davidson, 1976), in the HM7 human melanoma (Bhavanandan et al., 1981; Umemoto et al., 1981), and in MDA-MB-231 human metastatic mammary carcinoma cells in culture (Walker-Nasir et al., 1982). In the mouse mammary carcinoma and melanoma, and the human MDA-MB-231 cells, the O-glycoproteins were characterized by the probable presence of the same disaccharide chain (attached to serine or threonine) that is present in epiglycanin. The results suggest that a relationship exists between the presence of cell-surface O-glycoproteins and the capacity of tumor cells to escape the immune defense of the host. The radioimmunoassay with anti-epiglycanin antibody and $^{125}$I-epiglycanin described herein offers a means for investigating O-glycoproteins at the surfaces of tumor cells, particularly those with epiglycanin-related O-glycosyl-linked carbohydrate chains.

ACKNOWLEDGMENTS

The authors wish to thank Ms Debra Darby, Mr. James V. Ellard, Mrs. Lorna A. Lampert, Mr. Robert Perry, Mrs. Cyla Silber, and Mr. Kevin Sinclair for technical assistance. They are grateful to Dr. Agnes Schmit, RSCJ, for her collaboration.

Table I. Amounts of glycoproteins and neuraminidase-treated glycoproteins required to inhibit the reaction of $^{125}$I-epiglycanin with rabbit anti-epiglycanin antibody

| Glycoprotein | Activity detected | Amount (ng) for 50% inhibition[a] |
|---|---|---|
| Epiglycanin (Fr. II, ascites fluid) | ++++ | 2 |
| + Neuraminidase | ++++ | 0.5 |
| Epiglycanin (Fr. A, cleaved by protease | ++++ | 1.5 |
| + Neuraminidase | ++++ | 0.3 |
| ASGP-BI (Rat mammary carcinoma) | ++ | 1,700 |
| + Neuraminidase | ++ | 1,700 |
| ASGP-C1 (Rat mammary carcinoma) | ++ | 2,100 |
| + Neuraminidase | ++ | 2,000 |
| Carcinoembryonic antigen (CEA) | + | 40,000 |
| Bovine lubricating glycoprotein (LPG-I) | + | 5,000 |
| + Neuraminidase | ++ | >500 |
| Glycophorin | - | >5,000 |
| + Neuraminidase | + | >5,000[b] |
| Fetuin | - | >5,000 |
| + Neuraminidase | + | >5,000[b] |
| $\alpha_1$-Acid glycoprotein | - | >5,000 |
| + Neuraminidase | - | >5,000 |

| | | |
|---|---|---|
| Antifreeze glycoprotein | - | >5,000 |
| Porcine submaxillary mucin | + | >30,000[b] |
| Bovine submaxillary mucin | + | >30,000[b] |

[a]After incubating samples (50 µl) with anti-epiglycanin antiserum (100 µl, 1:1600), then incubating with $^{125}$I-epiglycanin (50 µl, 2 ng), they were incubated with normal rabbit serum (200 µl, 1:50) and goat anti-rabbit antiserum (150 µl). After incubation, the mixtures were centrifuged, and the radioactivities of the supernatants and residues determined. Values were calculated from a standard curve produced with epiglycanin.

[b]Partial (but less than 50%) inhibition was observed with the maximum amount tested.

Table II. Effect of various treatments on the inhibitory activity of epiglycanin in the radioimmunoassay

| Treated epiglycanin | Activity compared with untreated epiglycanin |
|---|---|
| Epiglycanin (Fr. II, untreated) | 100% |
| Periodate oxidation | 0% |
| Neuraminidase (V. cholerae) | 150-500% |
| TPCK-trypsin | 100-200% |
| N-Acetyl-α-D-galactosaminidase (C. lampas) | 100% |
| N-Acetyl-α-D-galactosaminidase (C. perfringens) | 100% |
| N-Acetyl-α-D-galactosaminidase (pig liver) | 100% |
| Endo-N-Acetyl-α-D-galactosaminidase (D. pneumonia) | 1% |
| 1. Neuraminidase (V. cholerae)[a] | 100% |
| 2. β-D-Galactosidase (C. ensiformis)[a] | 100% |
| 3. β-D-Hexosaminidase (C. ensiformis)[a] | 100% |
| 4. α-D-Mannosidase (C. lampas)[a] | 100% |

[a]Independent incubations were made with each enzyme. Also, epiglycanin was treated in the sequence 1-4 with each enzyme, and aliquots were tested after each incubation.

Table III. Capacity of various oligosaccharides or modified oligosaccharides present, or related to those present, in epiglycanin to inhibit the reaction of $^{125}$I-epiglycanin with rabbit anti-epiglycanin antiserum

| Substance tested | Amount required for 50% inhibition (ng) |
|---|---|
| β-D-Galp-(1→3)-D-GalNAc | >500 |
| β-D-Galp-(1→3)-α-D-GalNAc-(1→3)-Ser-Tosyl | >9,000 |
| β-D-Galp-(1→3)-α-D-GalNAc-(1→3)-Thr | >400,000 |
| β-D-Galp-(1→4)-D-GlcNAc | >5,000 |
| Me α-D-GalNAc | >5,000 |
| [β-D-Galp-(1→3)-α-D-GalNAc-(1→3)-Thr-Ala-Ala]$_n$ | >5,000 |
| β-D-Galp-(1→3)-D-GalNAc•ol | 43,000 |
| α-D-NeuNAc-(2→3)-β-D-Galp-(1→3)-D-GalNAc•ol | 33,000 |
| β-D-Galp-(1→4)-β-D-GlcNAcp-(1→3)-β-D-Galp-(1→3)-D-GalNAc•ol[a] | >18,000 |
| Glycopeptide "BH" | 16,000 |
| Asialoglycopeptide "BH" | 8,000 |

[a]Structure not rigorously established.

Table IV. Class of anti-epiglycanin antibody induced in the rabbit, as determined by precipitation with antibodies specific for the heavy chains of rabbit IgG, IgA, and IgM

| Anti-EPGN antiserum (1:150) (µl) | Goat-anti-rabbit IgA (α chain) (µl) | Goat anti-rabbit IgG (γ chain) (µl) | Goat anti-rabbit IgM (µ chain) (µl) | Buffer (µl) | Radioimmunoassay (% cpm in ppt.) | Loss of epiglycan binding activity |
|---|---|---|---|---|---|---|
| 100 | 100 | - | - | - | 63 | 13 |
| 100 | - | 100 | - | - | 45 | 56 |
| 100 | - | - | 100 | - | 46 | 55 |
| 100 | - | - | - | 100 | 67 | 0 |

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## LEGENDS TO FIGURES

Figure 1A: Induction of anti-epiglycanin antibody in two C57BL mice. An initial injection of $10^5$ TA3-Ha cells with Freund's complete adjuvant (▽, Day 0) was followed by injections (▼) of $10^5$ cells in the tail vein at approximately 7-day intervals. Blood was obtained at two-week intervals, and the sera tested for activity in the radioimmunoassay, as illustrated by the broken and solid bars. All sera were tested for antigen-binding activity at a dilution of 1:60.

Figure 1B: Induction of anti-epiglycanin antibody in one rabbit. An initial injection (▽, day 0) of $1.2 \times 10^7$ TA3-Ha ascites cells emulsified in Freund's complete adjuvant was followed by intravenous injections (▼) of $2 \times 10^6$ cells. A single intravenous injection of 120 µg of epiglycanin in PBS (○) was also given. Blood was obtained prior to injections, and the sera were compared for the presence of antigen-binding activity; all sera were tested at a dilution of 1:1600. Results are illustrated by the broken bars.

Figure 2. Binding of $^{125}$I-epiglycanin (2 ng, 50 µl) to mouse anti-epiglycanin antibody in 2-fold dilutions of the antiserum (100 µl). Normal mouse serum (100 µl, 1:50) and rabbit anti-mouse antiserum (75 µl) were added to coprecipitate the epiglycanin-anti-epiglycanin antibody complexes.

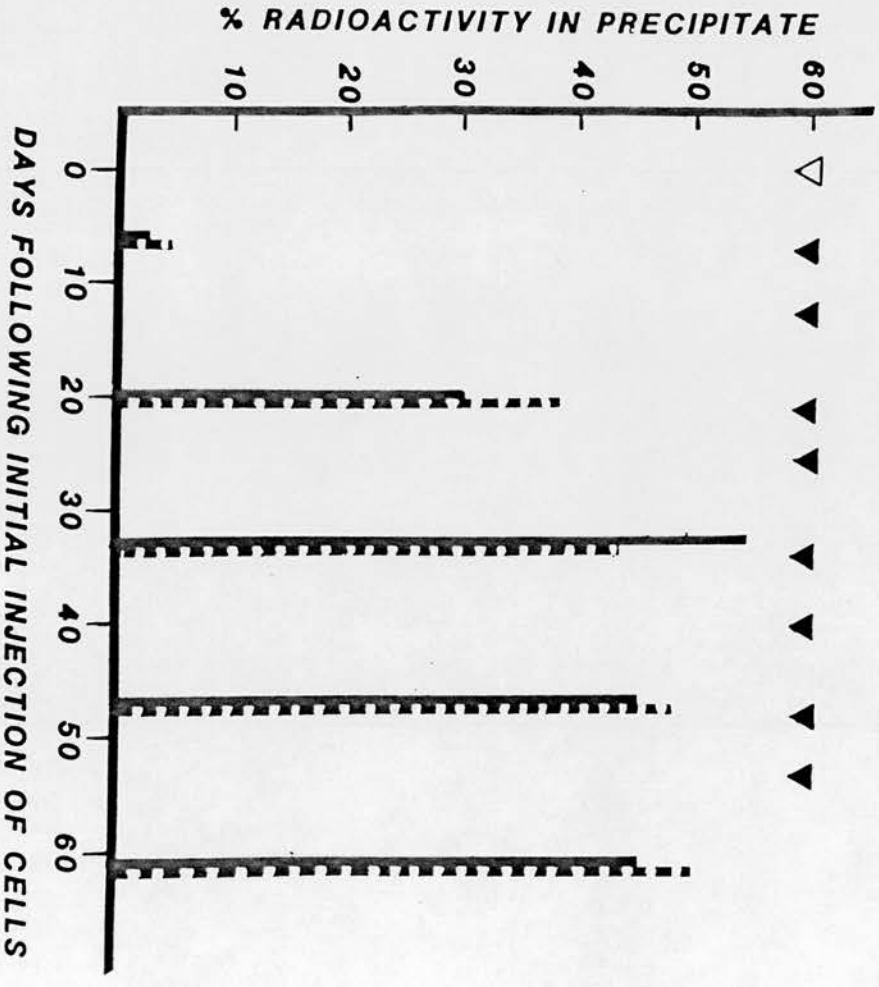
Figure 3. Binding of 3 ng of $^{125}$I-epiglycanin by two samples of rabbit anti-epiglycanin antiserum obtained at days 482 and 512 (see Figure 1B) is shown by the two curves. Normal rabbit serum and goat antirabbit antiserum were added to coprecipitate the epiglycanin-anti-epiglycanin antibody complexes.

Figure 4. Inhibition curves obtained by adding various amounts of epiglycanin in 50 µl volumes to 100 µl of rabbit anti-epiglycanin antiserum (1:1000; 0—0) or 100 µl of mouse anti-epiglycanin antiserum (1:60; Δ----Δ). In each experiment, approximately 2 ng of $^{125}$I-epiglycanin was used. The rabbit and mouse antiserums were tested with different batches of $^{125}$I-epiglycanin, which differed in specific activity.

Figure 5. Structure of the disaccharide chain: I, 2-acetamido-2-deoxy-3-O-β-D-galactopyranosyl-α-D-pyranose; II, attached to serine or threonine residues in the polypeptide chain. This glycopeptide structure appears to be part of the antigenic determinant against which anti-epiglycanin antibody is directed.

Figure 6. Fractionation of endo-N-acetyl-α-D-galactosaminidase-treated asialo-epiglycanin on a column (1.0 x 110 cm) of Bio-Gel P-100. Fractions (2.0 ml) were tested for inhibition of the binding of $^{125}$I-epiglycanin by anti-epiglycanin antiserum in the radioimmunoassay. The effluent volume of the untreated asialo epiglycanin is represented by a. b. Represents the effluent volume of the disaccharide (Fig. 5,I), as determined by a separate fractionation on the same column.

Figure 7. Gel filtration on a Sephadex G-200 column (1.0 x 111 cm) with 0.2 M sodium chloride as eluent. Upper figure (A): The first protein peak obtained by DEAE-cellulose chromatography of rabbit anti-epiglycanin antiserum (eluted with 0.01 M phosphate, pH 7.5). Lower figure (B): Fractionation on the same Sephadex G-200 column of material (peak IV) eluted from the DEAE-cellulose column with 1.0 M sodium chloride. Solid lines indicate protein concentration (mg/ml sample); dotted lines represent binding of $^{125}$I-epiglycanin.


% RADIOACTIVITY IN PRECIPITATE
10
20
30
40
50
60
DAYS FOLLOWING INITIAL INJECTION OF CELLS
0
10
20
30
40
50
60

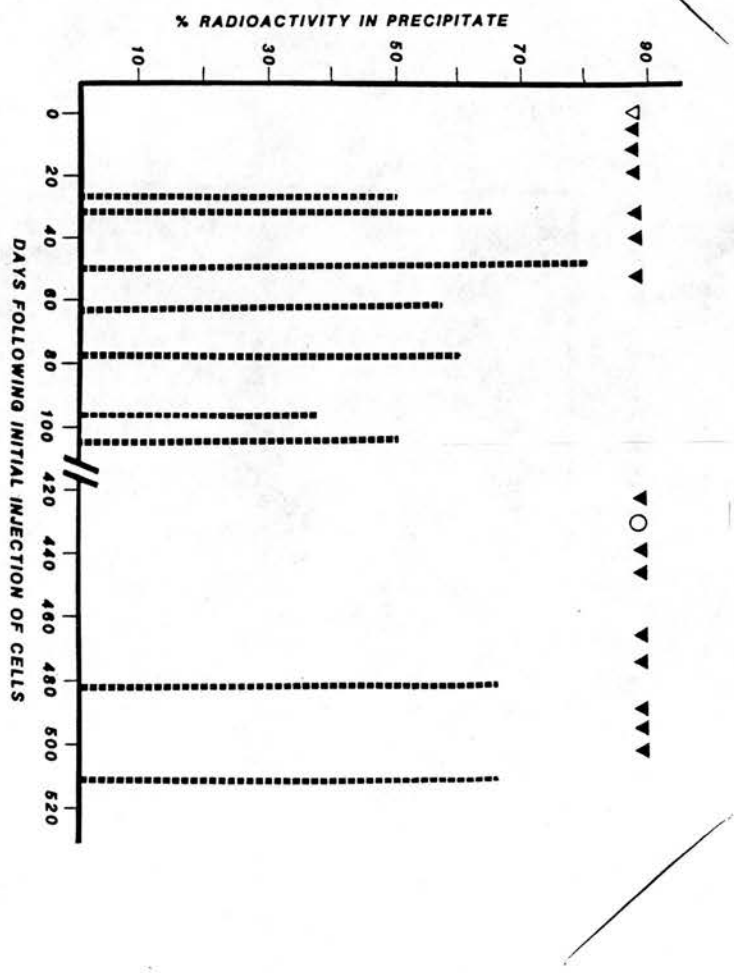

% RADIOACTIVITY IN PRECIPITATE
10
30
50
70
90
0
20
40
60
80
100
420
440
460
480
500
520
DAYS FOLLOWING INITIAL INJECTION OF CELLS

FIG. 13

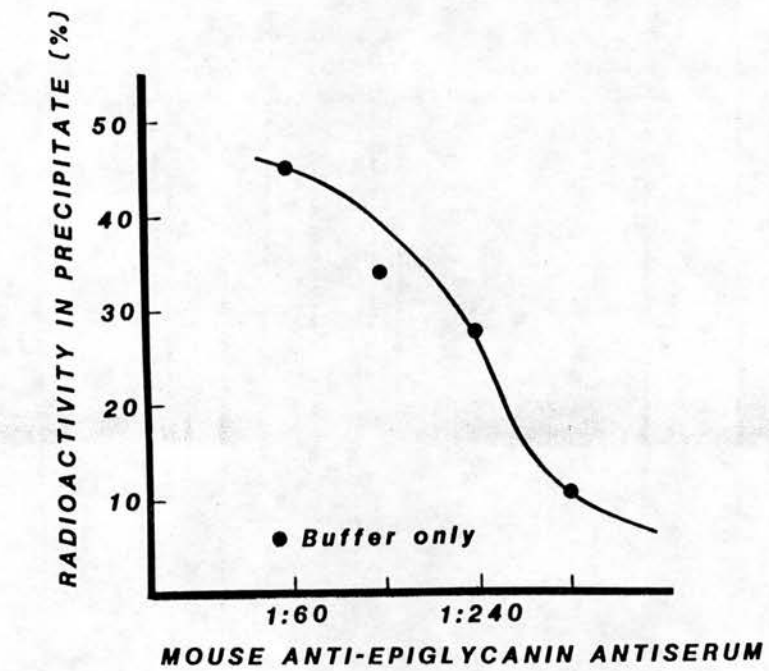

RADIOACTIVITY IN PRECIPITATE (%)
50
40
30
20
10
● Buffer only
1:60
1:240
MOUSE ANTI-EPIGLYCANIN ANTISERUM

FIG. 2

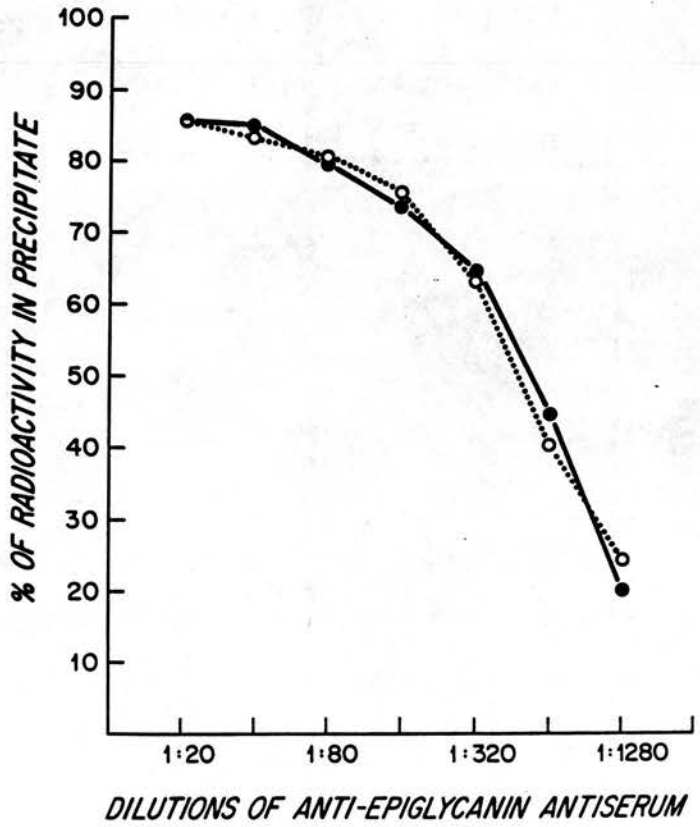

100
90
80
70
60
50
40
30
20
10
% OF RADIOACTIVITY IN PRECIPITATE
1:20
1:80
1:320
1:1280
DILUTIONS OF ANTI-EPIGLYCANIN ANTISERUM

FIG. 3

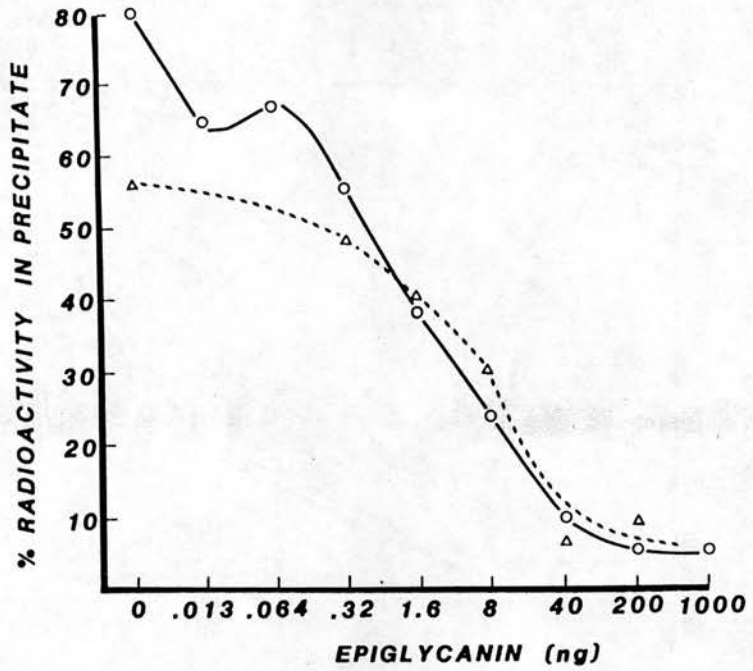

% RADIOACTIVITY IN PRECIPITATE
80
70
60
50
40
30
20
10
0 .013 .064 .32 1.6 8 40 200 1000
EPIGLYCANIN (ng)

FIG. 4

*I* $R = H$

*II* $R =$ *SERYL OR THREONYL*

FIG. 5

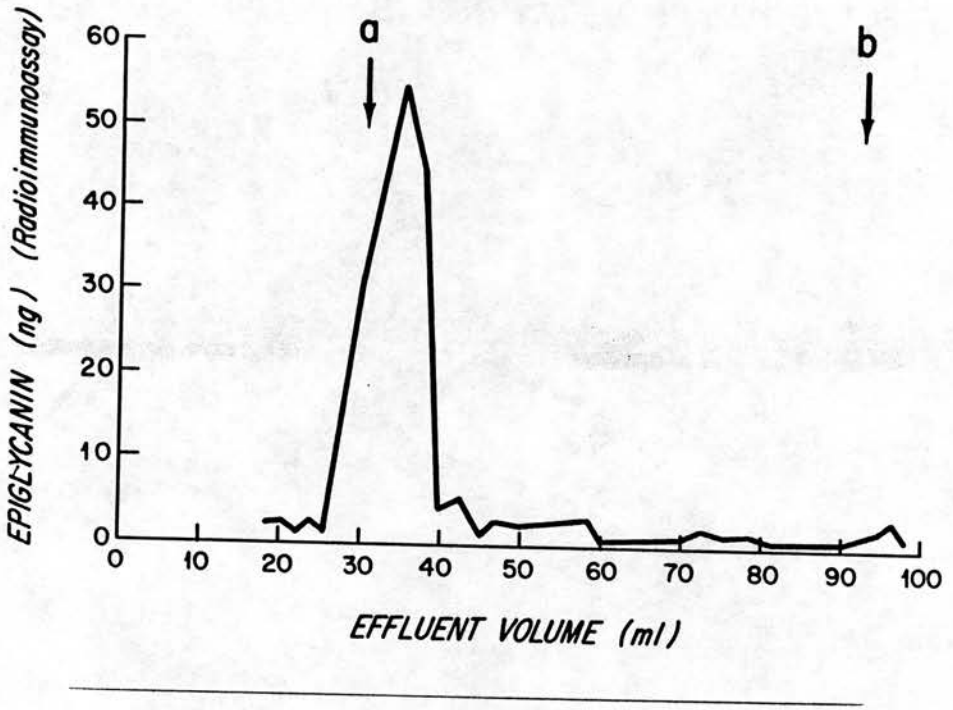

a
b
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
EPIGLYCANIN (ng) (Radioimmunoassay)
EFFLUENT VOLUME (ml)

FIG 6

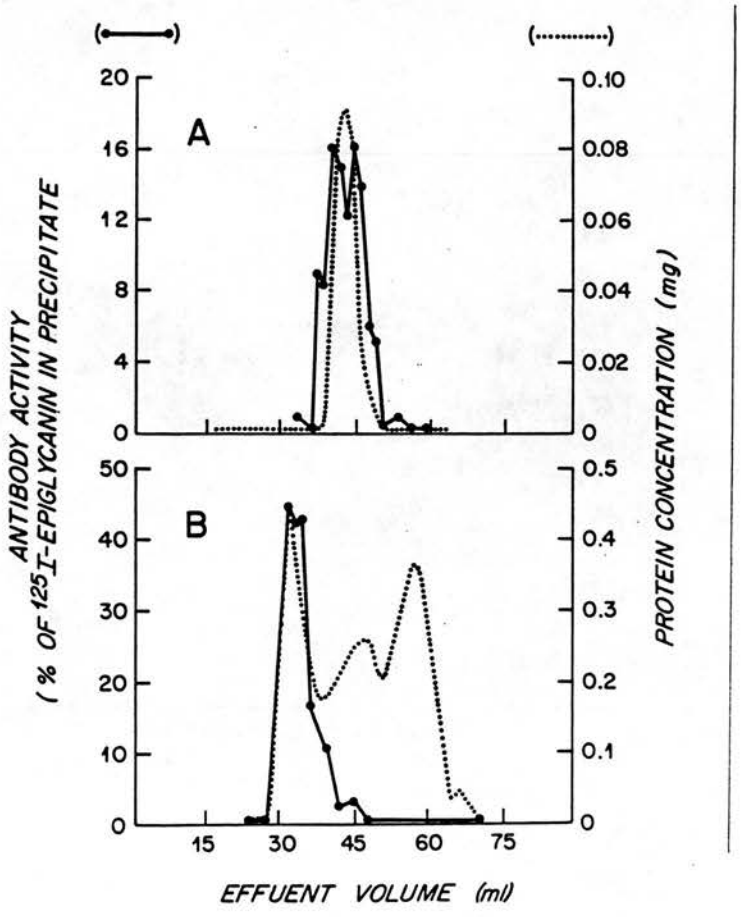

(——)
(·····)
A
B
20
16
12
8
4
0
0.10
0.08
0.06
0.04
0.02
0
50
40
30
20
10
0
0.5
0.4
0.3
0.2
0.1
0
15
30
45
60
75
ANTIBODY ACTIVITY
(% OF 125I-EPIGLYCANIN IN PRECIPITATE
PROTEIN CONCENTRATION (mg)
EFFUENT VOLUME (ml)

FIG. 7