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Glycoprotein Coat of the TA3 Cell. Isolation and Partial Characterization of a Sialic Acid Containing Glycoprotein Fraction†

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ABSTRACT: Sialic acid containing glycoprotein fragments were removed from the surface of the TA3 mammary adenocarcinoma ascites cell of the strain A mouse by short-term incubations with TPCK-trypsin at 4°. The material eluted in the void volume of a Bio-Gel P-100 column, after fractionation on Bio-Gels P-4 and P-30, represented 9% of the carbohydrate and protein material removed from the cells but 31% of the total surface sialic acid. It was composed of approximately 70% carbohydrate and 30% protein and contained four carbohydrate units, galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and sialic acid in the approximate molar proportion of 4:2:1:1. About 65 per 100 amino acid residues were serine and threonine. It contained two glycoprotein components having molecular weights of 88,000 and 180,000, as determined by elution from a column of Bio-Gel A-5m with 6 M guanidine hydrochloride, and 138,000 and 308,000 by sedimentation equilibrium.

It was recently suggested (Sanford and Codington, 1971) that the reduced transplantability of neuraminidase-treated TA3 mammary adenocarcinoma ascites cells of the strain A mouse in the foreign strain C3H mouse (Sanford, 1967) may be due to a serum factor which was demonstrated to be toxic *in vitro* to the cells after neuraminidase treatment. In a previous investigation of the sialic acid containing glycoproteins at the surface of this cell, Codington *et al.* (1970) demonstrated that peptide and carbohydrate material removed from the cells by chelating agents or proteolytic enzymes had widely different compositions, and all samples exhibited marked heterogeneity.

The mixture of glycoprotein fragments initially cleaved from the cells by mild treatment with TPCK-trypsin at 15–22° contained the highest proportion of sialic acid, suggesting that the glycoprotein structures richest in sialic acid were probably located at the periphery of the TA3 cell. This paper describes the fractionation by gel filtration of material obtained by short-term successive incubations with TPCK-trypsin at a lower temperature, 4°, and the partial characterization of the glycoprotein fraction having the highest proportion of sialic acid.

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Experimental Section and Results

Analytical Determinations. The protein content was determined by the Folin phenol reagent (Lowry *et al.*, 1951) with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., crystallized) as a standard, or calculated from the total amount of amino acid residues, as determined by an automatic amino acid analyzer. Amino acids were determined by a modification of the single-column method (Piez and Morris, 1960).

Sialic acid was measured by the thiobarbituric acid method (Warren, 1959) after mild acid hydrolysis or incubation with neuraminidase (EC 3.2.1.18, from *Vibrio cholerae*, Behringwerke, West Germany) with synthetic *N*-acetylneuraminic acid as standard. The proportion of *N*-acetyl- and *N*-glycolylneuraminic acids was determined on a Perkin-Elmer gas chromatograph, Model 900, OV 11 column (Supelco, Inc., Bellefonte, Pa.), as per(trimethylsilyl)ated methyl glycoside methyl esters, following methanolysis with 0.5 M methanolic hydrogen chloride for 60 min at 65°.

N-Acetylglucosamine, *N*-acetylgalactosamine, and neutral sugars were determined as their per(trimethylsilyl)ated methyl glycosides as follows. (A) Methanolysis with 0.5 M methanolic hydrogen chloride at 65° for 16 hr, followed by acetylation with acetic anhydride and pyridine, de-*O*-acetylation with

TABLE I: Material Isolated after Successive Incubations of TA3 Cells with TPCK-trypsin[a] at 4°.

Incubn	No. of Cells	% Viable Cells	Incubn Period (min)	Sialic Acid[b] (μg)	Protein (mg)
I	1.8×10^9	100	20	150	2.44
II	1.6×10^9	99	20	69	2.34
III	1.7×10^9	99	20	37	2.34
IV	1.8×10^9	98	20	40	2.34
V	1.7×10^9	99	20	9	2.52

[a] Concentration 18 μg/ml in balanced salt solution (Dulbecco and Vogt, 1954). [b] Determined by the thiobarbituric acid method (Warren, 1959).

TABLE II: Relative Molar Quantities of Carbohydrate Components[a] in Nondialyzable Material Released from 1.7×10^9 TA3 Cells by TPCK-trypsin.[b]

Incubation	GalNAc/Gal	GNAc/Gal	Sialic Acid/Gal	Gal (μg)
I	0.59	0.39	0.33	253
III	0.52	0.49	0.30	69
IV	0.54	0.50	0.42	47
V	0.64	0.90	0.24	21

[a] Determined by gas–liquid chromatography (method A). [b] Results for this experiment are reported in Table I.

methanolic ammonia, and per(trimethylsilyl)ation (Reinhold 1972). This method gives reliable sialic acid values but fails to cleave all 2-acetamido-2-deoxygalactosyl linkages, thus resulting in slightly low *N*-acetylgalactosamine values. (B) Hydrolysis with 2.0 M trifluoroacetic acid at 120° for 2 hr, followed by methyl glycosidation and the same procedure as outlined under A. Method B results in the loss of the sialic acid but gives reliable *N*-acetylglucosamine and *N*-acetylgalactosamine values. Values determined by gas–liquid chromatography are accurate within 10–15%.

Treatment of Cells with Trypsin. The TA3 mammary adenocarcinoma ascites cells of the strain A mouse (Hauschka, 1953; Sanford, 1967) were isolated, freed of erythrocytes, and washed three to six times with a balanced salt solution (Dulbecco and Vogt, 1954), as previously described (Codington *et al.*, 1970). Temperatures of 0–4° and sterile techniques were maintained in all cell experiments. The procedure of incubation with TPCK-trypsin previously described (Codington *et al.*, 1970) was modified to involve a larger number of incubations and a lower temperature (0–4°), resulting in less loss of cell viability.

Incubation of each batch of cells ($1–3 \times 10^9$ cells) with TPCK-trypsin (18 μg/ml) in a 20-ml suspension of balanced salt solution was performed for approximately 20 min with 60–80 rotations/min on a Thomas rotating apparatus. After five to seven incubations had been completed, the volume of each supernatant solution was measured. A small amount of cell debris was removed by centrifugation at about 20,000*g*, and each solution was lyophilized separately. Two series of experiments, one (series I) utilizing 5.4×10^{10} TA3 cells in twelve separate batches of cells, and the other (series II), 2.0×10^{10} cells in eight separate batches, were performed. The material cleaved from the cells in each incubation in the series I experiments was analyzed for its carbohydrate and amino acid composition, as described below. In series II, the material obtained in the several incubations was pooled.

The results of one series of incubations are presented in Table I. The proportion of sialic acid in the material removed from the cells decreases markedly as the proteolysis proceeds; whereas, the protein content remains relatively constant. This procedure removed 40–50% of the surface sialic acid residues, the value of which was found to be 0.44 mg/10^9 cells (Codington *et al.*, 1970).

In order to determine the proportion of carbohydrate components in the nondialyzable part of the material removed from the cells, an aqueous solution of the residue was dialyzed against water, and the retentate analyzed by gas–liquid chromatography (method A). These results are presented in Table II. Both mannose and glucose were detected in significant amounts, as well as galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and sialic acid. In a control experiment of the same dialysis conditions, significant amounts of glucose and mannose were released from the dialysis tubing and found in the retentate after lyophilization. Although both carbohydrates have been detected in nondialyzed material obtained under the same proteolysis conditions, the accuracy of the mannose and glucose values found in this experiment is dubious, and these values are omitted from Table II. Because of the probability of contamination of cell derived material by dialysis tubing, dialysis was not used for any other material described in this paper. The amount of protein and carbohydrate material removed during five to seven incubations with the enzyme, as determined by colorimetric procedures, represented 4–6% of the dry weight of the cells (9–13 mg/10^9 cells).

Fractionation of Released Material by Gel Filtration. Gel filtrations were performed on Bio-Gels P-4, P-30, and P-100, 100–200 mesh, at 4° with 0.05 M pyridine acetate (pH 5.3) as eluent. The void volume of each column was determined with Blue Dextran 2000 (Pharmacia, Uppsala, Sweden).

Fractionation on Bio-Gel P-4. All samples from a given incubation period in series I were pooled and fractionated on a column of Bio-Gel P-4. The fractionation of the material obtained from incubation I, expressed as the protein and sialic acid content of the column effluent, is presented in Figure 1. Fractionation of the pooled material from the other six incubations gave a similar profile showing two major protein peaks. The amount of sialic acid is relatively smaller, and the second protein peak relatively greater in each succeeding incubation with trypsin. For each incubation, the greatest proportion of sialic acid, as determined by the thiobarbituric acid method after acid hydrolysis, was found in the material eluted in the void volume (peak I). A similar elution profile was found for the material pooled from all successive incubations (series II). The first peak (series I) was divided into two fractions, I_A and I_B, which were analyzed separately. The highest proportion of sialic acid was always found in fraction I_A, and fraction I_B was not further investigated. Examination of fraction I_A (Table III) shows that the proportion of sialic acid to protein decreases as the trypsinization proceeds. The major components were found to be galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and sialic acid. The relative

TABLE III: Composition of Fraction I_A Eluted from a Column of Bio-Gel P-4 (Series I).

Components	Rel Molar Proportions[a]	
	Incubn I	Incubn V
Mannose/galactose	0.03	0.03
N-Acetylgalactosamine/galactose	0.46	0.44
N-Acetylglucosamine/galactose	0.14	0.18
Sialic Acid/galactose	0.20	0.26
Sialic Acid/protein[b]	0.07	0.02
Amino Acids	**Residues/1000 Residues**	
Aspartic acid	61	103
Threonine	121	95
Serine	111	104
Glutamic acid	75	137
Proline	104	56
Glycine	108	82
Alanine	114	81
Cysteine	10	38
Valine	59	53
Methionine	11	22
Isoleucine	38	46
Leucine	75	62
Tyrosine	19	17
Phenylalanine	25	27
Lysine	28	47
Histidine	16	13
Arginine	23	18

[a] Carbohydrate components determined by gas–liquid chromatography; protein determined colorimetrically. [b] Moles of protein based upon a value of 104 for an average amino acid residue (Codington *et al.*, 1970).

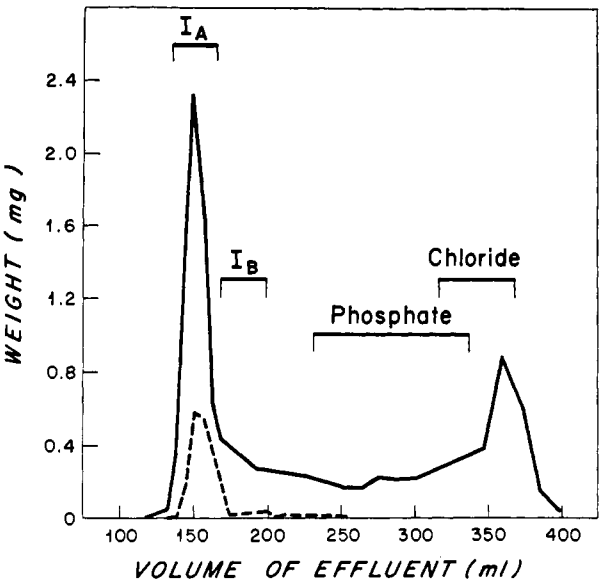

FIGURE 1: Fractionation at 4° of material (series I) cleaved by TPCK-trypsin from 5.4×10^{10} TA3 cells on a Bio-Gel P-4 column (2.4×80 cm). Eluent, pyridine acetate, 0.05 M, pH 5.3. Solid line, protein; broken line, sialic acid. Incubation I.

proportion of the carbohydrate components remains nearly constant in the fractions I_A from the first through the fifth incubation. A comparison of the molar ratios of the carbohydrate components before (Table II) and after fractionation (Table III) reveals that the gel filtration gave a fraction (fraction I_A) enriched in galactose but having a lower proportion of *N*-acetylglucosamine relative to *N*-acetylgalactosamine.

The amino acid compositions of fractions I_A (Table III) show a particularly large proportion of hydroxyamino acids, and of aspartic and glutamic acids. By comparison, fractions I_B contain proportionately less serine and threonine and more aspartic and glutamic acids.

Fractionation on Bio-Gel P-30. Because of the differences observed in the relative proportions of protein and carbohydrate material removed in successive incubations, as well as in the amino acid compositions of the protein moiety (Table III), the fractions I_A from the first series of experiments (series I) were not pooled. Each of the seven samples was further fractionated separately on a column of Bio-Gel P-30. The major characteristics of the elution profiles, based upon both protein and sialic acid determinations, were similar for the materials obtained from each of the seven incubations and also similar to that given by the pooled material (series II), shown in Figure 2. Sharp peaks representing both protein and sialic acid content are located at the void volume, and the remaining fractions contain only small or undetectable amounts of the latter component. The carbohydrate and amino acid compositions of material in the major peak, peak I, from all incubations remained relatively constant through the seventh incubation. This material was characterized by a high content of galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and *N*-acetylneuraminic acid, and an exceptionally large proportion of the hydroxyamino acids, serine and threonine. The proportion of mannose was very small, and fucose was not detected.

Filtration on Bio-Gel P-100. Part of the material (17.6 mg)

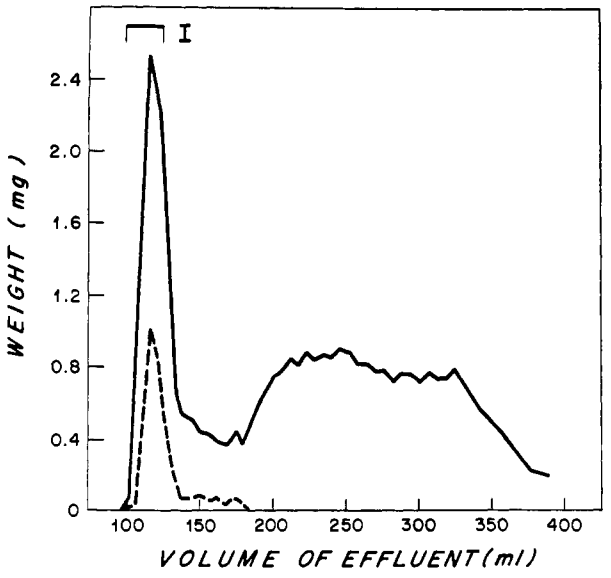

FIGURE 2: Fractionation at 4° of material from a column of Bio-Gel P-4, Fraction I_A, on a Bio-Gel P-30 column (2.5×70 cm). Eluent, pyridine acetate, 0.05 M, pH 5.3. Solid line, protein; broken line, sialic acid. Pooled sample (57.8 mg), series II, from 2.0×10^{10} cells.

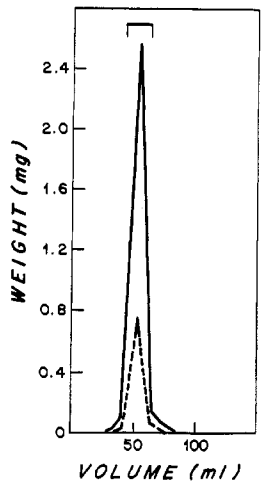

FIGURE 3: Fractionation at 4° of 17.6 mg of material (series II) from a column of Bio-Gel P-30, peak I (see Figure 2), on a Bio-Gel P-100 column (2.7 × 40 cm). Eluent, pyridine acetate, 0.05 M, pH 5.3. Solid line, protein; broken line, sialic acid.

eluted at the void volume, peak I, from a Bio-Gel P-30 fractionation of the pooled samples (series II) was applied to a column of Bio-Gel P-100 (2.7 × 40 cm). All detectable material was eluted from the column in a single symmetrical peak within the void volume to give 15.7 mg (89% recovery) of material after lyophilization (Figure 3). This fraction is designated glycoprotein fraction I.

An estimate of the amount of this material present on the TA3 cell surface is based upon the yield of the isolated fraction. The supernatant solutions obtained after incubation of the cells with TPCK-trypsin contained an average of 11 mg of carbohydrate and protein material from 10^9 cells for both series I, 5.4×10^{10} cells, and series II, 2.0×10^{10} cells. The weight of fractions I_A, isolated from the effluents from the P-4 columns, varied, however, 2.0 mg/10^9 cells in series I and 3.0 mg in series II. Seven fractionations on Bio-Gel P-30 in series I gave a total of 0.65 mg of glycoprotein/10^9 cells compared to a higher value, 1.2 mg/10^9 cells, for material obtained in a single fractionation of a pooled sample in series II. The recovery of material from the fractionation on Bio-Gel P-100 in series II was 21 mg of glycoprotein fraction I, 1.05 mg/10^9 cells. This represents 9.4% of the protein and carbohydrate material removed from the cells by the enzyme (series II). The sialic acid content (2.8 mg) of this fraction was 13%. This is 31% of the total TA3 cell surface sialic acid (Codington *et al.*, 1970). Since this figure is based upon isolated material, it appears probable that it represents a minimal figure, and that as much as 40–50% of the sialic acid at the surface of the TA3 cell may be attached to glycoprotein in this fraction.

The composition of the material obtained by fractionation on Bio-Gel P-100 (Table IV) shows little difference from that of material eluted in peak I from a column of Bio-Gel P-30 (Figure 2). Galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and *N*-acetylneuraminic acid represent 27, 19, 9, and 13%, respectively, of the fraction, for a total of 68%. *N*-Glycolylneuraminic acid, which constitutes about 10% of the sialic acid of the TA3 cell surface, is absent. Residues of proline, alanine, and glycine are present in abundance, but very few sulfur-containing, aromatic or basic amino acids are present. Only 9 residues of aspartic acid per 1000 residues were detected.

TABLE IV: Composition of Glycoprotein Fraction I Eluted from a Column of Bio-Gel P-100.

Component	%
Protein	31[a]
Galactose	27[b]
Mannose	0.5[b]
N-Acetylgalactosamine	19[b]
N-Acetylglucosamine	9[b]
N-Acetylneuraminic acid	13[a], 12[c]
Amino Acid[d]	**Residues/1000 Residues**
Aspartic acid	9
Threonine	367
Serine	281
Glutamic acid	13
Proline	114
Glycine	84
Alanine	106
Valine	7
Isoleucine	3
Leucine	18

[a] Determined by colorimetric procedures. [b] Determined by gas–liquid chromatography (method B). [c] Method A. [d] Residues not listed were present only in trace amounts or were not detected.

Molecular Weight Determination of Glycoprotein Fraction I by Gel Filtration in 6 M *Guanidine Hydrochloride Solution.* The *N*-acetylneuraminic acid residues of a sample (860 μg, containing 0.36 μmole of *N*-acetylneuraminic acid) were labeled with tritium at carbon-7 by the method of Lenten and Ashwell (1971). A column of Bio-Gel P-100 (2.2 × 84 cm) was charged with a sample of labelled material, which was then eluted with pyridine acetate (50 mM, pH 5.3). Radioactivity in counts per minute was determined with a Packard Tri-Carb scintillation counter. The plot of the radioactivity of the collected fractions *vs.* the effluent volume gave a single peak identical with that obtained by plotting the protein content of the nonlabelled material (Figure 3). The fractions containing radioactivity were lyophilized.

The residue was dissolved in 6 M guanidine hydrochloride (3 ml) containing 1 mM cysteine and each of the following proteins as standards: myosin (chicken leg, chromatographically purified, mol wt 200,000, 8 mg), bovine serum albumin (Sigma Chemical Co., mol wt 69,000, 8 mg), and lysozyme (General Biochemicals, Chagrin Falls, Ohio, three-times crystallized, mol wt 14,300, 4 mg). The solution was applied to a column of Bio-Gel A-5m (1.9 × 90 cm), and the fractionation performed essentially as described by Fish *et al.* (1969).

Elution with 6 M guanidine hydrochloride and 1 mM cysteine readily separated the standard proteins (Figure 4). In one experiment the effluent volumes showed a straight line when plotted against the log of their molecular weights, in accordance with the equation relating the radius of gyration and the molecular weight (Fish *et al.*, 1969). In a second experiment the three points were not linear. Two peaks of radioactivity were observed. These were eluted between myosin and bovine serum albumin in each case. From a plot of the molec-

ular weights versus effluent volumes, the average apparent molecular weights were found to be 88,000 and 180,000.

Disc Gel Electrophoresis. By polyacrylamide gel electrophoresis (5% gel) with 0.1% sodium dodecyl sulfate (Shapiro *et al.*, 1967) only one broad, but faint, band was observed for glycoprotein fraction I after periodate–Schiff staining (Keyser, 1967). By comparison with standard proteins the approximate molecular weight was estimated to be in the 150,000–200,000 range.

Molecular Weight Determination by Sedimentation Equilibrium. A sample of glycoprotein fraction I (360 μg) in a solution (0.3 ml) of 6 M guanidine hydrochloride and 0.01 M EDTA at pH 7.0 was dialyzed against the same solution for 48 hr at 4°. The material was analyzed by the high-speed method of Yphantis (1964) in a Spinco Model E analytical ultracentrifuge. The partial specific volume ($\bar{v}$, 0.659) was calculated from published values for carbohydrate and amino acid components. The results are consistent with the presence of two components, according to the method described by Roark and Yphantis (1969). The molecular weights determined by this method were 1.6–1.7 times greater than those obtained by gel filtration. The smaller component was found to have a molecular weight of 138,000 (±5%). The molecular weight of the second component was calculated to be 308,000 (±10%).

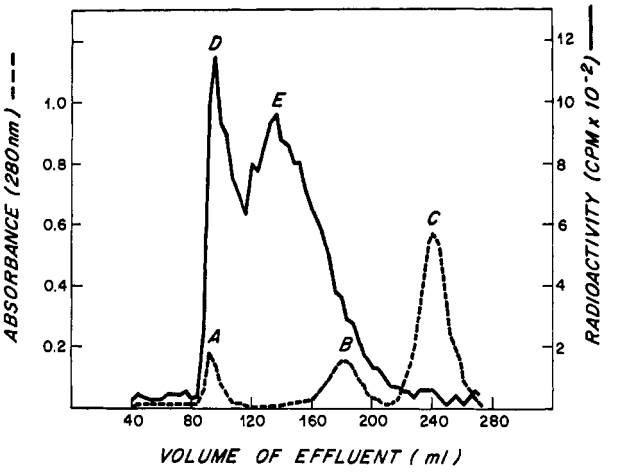

FIGURE 4: Fractionation at 4° of ^{3}H-labeled glycoprotein fraction I (series II) from a Bio-Gel P-100 column (see Figure 3) and proteins of known molecular weight on a Bio-Gel A-5m column (1.9 × 90 cm). Eluent, guanidine hydrochloride, 6 M, and cysteine, 1 mM. Broken line, absorbance at 280 nm; solid line, radioactivity in counts per minute. Proteins: A, myosin; B, bovine serum albumin; C, lysozyme; D and E, glycoprotein fraction I.

Discussion

Isolation from the TA3 cell surface of a glycoprotein fraction having a high sialic acid content (glycoprotein fraction I) was achieved by successive passages through columns of Bio-Gels P-4, P-30, and P-100. Based upon the elution profile (Figure 4) obtained by passage of a labeled sample through a column of Bio-Gel A-5m in 6 M guanidine hydrochloride (Fish *et al.*, 1969) and sedimentation equilibrium (Yphantis, 1964; Roark and Yphantis, 1969), glycoprotein fraction I was shown to be a mixture of two high molecular weight components or families of components. The two glycoproteins appeared to be present in approximately equal amounts. The molecular weight range of the single broad band, approximately 150,000–200,000, obtained by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (Shapiro *et al.*, 1967) was not markedly different from values obtained by gel filtration, 88,000 and 180,000, and those calculated from sedimentation equilibrium data, 138,000 and 308,000. Yet, Segrest *et al.* (1971) recently reported that the molecular weights of glycoproteins obtained by gel electrophoresis are too high. It is interesting that α_1-acid glycoprotein, having a carbohydrate content of 40%, gave in our hands a correct molecular weight, 41,000, by gel filtration. These apparent inconsistencies may be due to the differences in both composition and structure of glycoproteins.

Glycoprotein fraction I was released from the cells by the action of TPCK-trypsin at 0–4°. The low number of peptide bonds cleaved by the enzyme may be due to the unusually large number of negatively charged sialic acid end groups (Yamashina, 1956; Gottschalk *et al.*, 1960), which are spaced along the protein core at an average of one for every six amino acid residues, or more probably to the absence of a significant number of basic amino acid residues (Table III).

In contrast to the observations of Kraemer (1971), TPCK-trypsin removed initially components containing the largest proportion of sialic acid groups from the TA3 cells, which suggests (Codington *et al.*, 1970) that structures having the largest proportion of sialic acid groups are located at the extreme periphery of the cell. The small variation in the relative proportions of carbohydrate components and amino acid residues in the fractionated material through successive incubations (series I) suggests that the two components of glycoprotein fraction I may be fragments of the same large glycoprotein or of related glycoproteins which are rooted in the cell membrane in a manner similar to that proposed for the M and N blood group substances on the surface of the erythrocyte (Morawiecki, 1964). The relatively large amount of carbohydrate material, 70%, in the molar proportions of 4:2:1:1 for galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and *N*-acetylneuraminic acid, respectively, suggests that these glycoproteins are of a structure related to that of secreted glycoproteins of the mucin type. Consistent with this type of structure is the presence of a large proportion of hydroxyamino acids, which represent 65% of all amino acid residues. Serine and threonine are known to be involved in glycopeptide linkages in mucin-type glycoproteins. The large percentage of sialic acid, about 13%, suggests the presence of a large number of carbohydrate chains, since these groups would normally occupy terminal positions.

No material having a quantitative composition similar to that of glycoprotein fraction I has been reported as yet. However, some of the material cleaved by trypsin incubation of a glycoprotein of the erythrocyte stroma (Winzler *et al.*, 1967; Winzler, 1970) contains the same four carbohydrate components, although in different proportions. The composition of glycoprotein fraction I bears little resemblance to that of other glycoproteins isolated from tumor cell surfaces after proteolysis (Walborg *et al.*, 1969; Shen and Ginsburg, 1968; Langley and Ambrose, 1967) or to glycoproteins having histocompatibility antigen activity isolated from mammalian cell membranes (Davies, 1970; Nathenson *et al.*, 1970; Sanderson *et al.*, 1971), which contain fucose, a sugar commonly detected in isolated membranes from mammalian cells (Buck *et al.*, 1970). The absence of fucose in the material released by TPCK-trypsin from TA3 cells suggests either that the fucose-containing glycoproteins are not susceptible to attack by TPCK-trypsin or that the enzyme is not penetrating into the cell's outer membrane.

Isolation procedures described in this study produced 1.05

mg of glycoprotein fraction I from 10^9 cells. This represents 31% of the sialic acid of the cell surface. Based upon the topography of the TA3 cell, as observed by electron microscopy (Codington *et al.*, 1970), there are approximately 2.0×10^5 sialic acid residues bound to glycoprotein fraction I per μ^2. The size of the native glycoprotein molecule or molecules is not yet known, but on the basis of an average molecular weight of 223,000, as determined by sedimentation equilibrium, approximately 2.0×10^3 molecules of this material of an average length of more than 2000 Å, if in an extended configuration (Morawiecki, 1964), are present per μ^2 of the cell surface, resulting in an average spacing of less than 300 Å. The material isolated in this study may represent only a fragment of the native glycoprotein. The larger parent macromolecules would be spaced at greater distances but would have greater length and extend correspondingly greater distances from the cell membrane. It might be expected that this fraction, abundant on the surface of the TA3 cell, could play a significant role in the malignant processes of this cell.

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