References

Adler, F. L. (1964), *Progr. Allergy 8*, 41.
Amkraut, A. A., Garvey, S. J., and Campbell, D. H. (1966), *J. Exp. Med. 124*, 293.
Becker, M., Conway-Jacobs, A., Wilchek, M., Haimovich, J., and Sela, M. (1970), *Immunochemistry 7*, 741.
Borek, F., and Stupp, Y. (1966), *Immunochemistry 3*, 339.
Conway-Jacobs, A., Schechter, B., and Sela, M. (1970), *Israel J. Med. Sci. 6*, 440.
Crumpton, M. J. (1966), *Biochem. J. 100*, 223.
Crumpton, M. J., and Small, P. A., Jr. (1967), *J. Mol. Biol. 26*, 143.
Djerassi, C. (1960), Optical Rotatory Dispersion, New York, N. Y., McGraw-Hill.
Fairclough, G. F., Jr., and Vallee, B. L. (1969), 158th National Meeting of the American Chemical Society, New York, N. Y., Abstract No. BIOL 248.
Haurowitz, F. (1936), *Hoppe-Seyler's Z. Physiol. Chem. 245*, 23.
Hofsten, B. V., and Falkbring, S. O. (1961), *Anal. Biochem. 1*, 437.
Hooker, S. D., and Boyd, W. C. (1932), *J. Immunol. 23*, 465.
Kagan, H. M., and Vallee, B. L. (1969), *Biochemistry 8*, 4223.
Landsteiner, K. (1945), The Specificity of Serological Reactions, rev ed, Cambridge, Mass., Harvard University.
Leskowitz, S. (1963), *J. Exp. Med. 117*, 909.
Merz, W. (1959), *Mikrochem. Acta 4*, 640.
Ouchterlony, O. (1953), *Acta Pathol. Microbiol. Scand. 32*, 231.
Porter, R. R. (1959), *Biochem. J. 73*, 119.
Pressman, D., and Grossberg, A. (1968), The Structural Basis of Antibody Specificity, New York, N. Y., Benjamin.
Ramachandran, J. (1967), *7th Intern. Congr. Biochem. (Tokyo)*, J-144, 982.
Robbins, J. B., Haimovich, J., and Sela, M. (1967), *Immunochemistry 4*, 11.
Schechter, I., Schechter, B., and Sela, M. (1966), *Biochim. Biophys. Acta 127*, 438.
Sela, M., Fuchs, S., and Arnon, R. (1962), *Biochem. J. 85*, 223.
Sela, M., Schechter, B., Schechter, I., and Borek, F. (1967), *Cold Spring Harbor Symp. Quant. Biol. 32*, 537.
Slobin, L. (1970), *Nature (London) 225*, 698.
Tabachnick, M., and Sobotka, H. (1959), *J. Biol. Chem. 234*, 1726.
Vallee, B. L. (1970), *8th Intern. Congr. Biochem., Interlaken, Switzerland*, 56.

Studies on the Carbohydrate Portion of Membrane-Located Mouse *H-2* Alloantigens*

Takashi Muramatsu and Stanley G. Nathenson

ABSTRACT: Membrane-located *Histocompatibility-2* (*H-2*) alloantigens labeled with radioactive sugars were solubilized by papain digestion and purified from three types of murine tumors: MTC (mastocytoma, $H\text{-}2^d$), Meth-A (fibrosarcoma, $H\text{-}2^d$), and EL-4 (lymphoma, $H\text{-}2^b$). An antibody–antigen binding reaction between the *H-2* alloantigen and alloantibody was utilized as the final purification step. It was established that galactose, mannose, fucose, glucosamine, and sialic acid are integral components of immunologically active *H-2* alloantigen glycoproteins of tumor cells of all *H-2* strains examined. About 4–6% of [^{3}H]fucose and somewhat smaller proportions of the other sugars that were incorporated into crude membranes were present in the papain-solubilized *H-2* alloantigens. Radioactive glycopeptides prepared by pronase digestion of carbohydrate-labeled *H-2* alloantigens from the three tumor cells formed single sharp peaks upon Sephadex G-50 column chromatography and each showed an elution volume corresponding to a molecular weight of 3300. In contrast to the high degree of homogeneity of *H-2* glycopeptides with respect to size, glycopeptides prepared from crude membranes or from cell surfaces formed broad peaks upon Sephadex G-50 column chromatography. Upon DEAE-Sephadex column chromatography, the *H-2* glycopeptides from Meth-A cells ($H\text{-}2^d$) or EL-4 cells ($H\text{-}2^b$) were separated into two major sharp peaks. The chromatographic double-label patterns were almost identical when preparations from each source were mixed before chromatography. Glycopeptides from crude membrane preparations of the two tumor cells formed broad peaks upon DEAE-Sephadex column chromatography.

These results demonstrate the close similarities of the *H-2* glycopeptides from the two tumor cells differing both in cell type and *H-2* specificity profile and the unique properties of *H-2* glycopeptides with respect to size and charge when compared to the heterogeneous array of glycopeptides from the whole membrane of these tumor cells.

The *histocompatibility-2* (*H-2*) alloantigens are the major transplantation antigens of the mouse, and are located on the cell surface membrane. The *H-2* alloantigens have been solubilized from their membrane site by papain digestion and purified by a series of fractionation procedures. The purified materials were found to be glycoproteins containing

* From the Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461. *Received June 22, 1970.* This research was supported in part by Grant AI-07289 from the U. S. Public Health Service and Grant GB-7924 from the National Science Foundation. S. G. N. is a recipient of a research development award from the U. S. Public Health Service (AI-11569).

TABLE I: Recovery of Radioactivity During the Purification of *H-2* Alloantigen.

Radioactive Sugars	Type of Tumor Cells	% Radioactivity Incorpd into Cells	% Radioactivity in Cells Recovd in Crude Membranes	% Radioactivity in Crude Membranes Estimated to Be Present in *H-2* Alloantigen[a]
[^{3}H]Fucose	MTC	9.6	32	6.2
	Meth-A	11.0	35	4.3
	EL-4	8.5	29	6.2
[^{3}H]Glucosamine	MTC	12.0	41	1.4
	Meth-A	12.0	34	2.2
[^{14}C]Glucosamine	EL-4	13.0	32	1.8
[^{3}H]Galactose	Meth-A	10.0	30	1.1
	EL-4	11.0	29	1.5
[^{14}C]Mannose	Meth-A	5.5	33	1.6
	EL-4	7.0	28	1.5

[a] These figures were calculated from actual data by correcting for recovery of *H-2* alloantigenic activity during partial purification (MTC, 9%; Meth-A, 13%; EL-4, 12%) and recovery of antigen–antibody complex after gel filtration (70%). Thus, the total amount of radiolabeled antigen which complexed with antibody was corrected for only a 70% recovery at complex isolation stage, and for an approximate recovery during purification (*e.g.*, for Meth-A, 13% of the activity on membranes was recovered in the Sephadex G-150 stage).

about 10% carbohydrate (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970a). The availability of these purified glycoproteins offered an unique opportunity to examine the chemical architecture of the carbohydrate moieties of a documented membrane-located macromolecule. Such information should reveal features shared generally by membrane-located glycoproteins, and also contribute to an understanding of the *H-2* immunological specificities in molecular terms. Because of the very small quantities of purified *H-2* alloantigens available, we have prepared these alloantigens radiolabeled in their carbohydrate moieties from cultured tumor cells grown in the presence of radioactive monosaccharides by methods previously reported (Muramatsu and Nathenson, 1970a). In this paper we describe in detail the properties of carbohydrate-labeled glycopeptides isolated from three cell lines of two *H-2* genotypes.[1]

Materials and Methods

Mice, Alloantiserum, and Test System. Mouse strains and preparation of alloantiserum have been described previously (Shimada and Nathenson, 1969). Alloantigenic activity was detected as described previously (Nathenson and Davies, 1966) by the method of inhibition of immune cytolysis (Sanderson, 1964; Wigzell, 1965). The IgG fractions of alloantisera were purified by ammonium sulfate fractionation, Sephadex G-200 column chromatography, and disc gel electrophoresis (the preparation used for experiments with MTC cells was a gift of Dr. A. Shimada), or by Sephadex G-200 column chromatography alone.

Incorporation of Radioactive Sugars into Cultured Tumor Cells. The MTC-cell line (*H-2^d*, Mastocytoma P815) was a gift from Dr. Ted Brunner, Lausanne, Switzerland, and was maintained in DBA/2 mice. Meth-A cells (*H-2^d*, fibrosarcoma) and EL-4 cells (*H-2^b*, lymphoma) were obtained from Dr. F. Lilly, Albert Einstein College of Medicine, and were maintained in BALB/c mice and C57BL/6 mice, respectively. These tumor cells were cultured directly from the peritoneum in spinner suspension at 37° in 500–1000 ml of culture medium. MTC-cells (1×10^6 cells/ml) were cultured in minimum essential medium (Joklik-modified, Grand Island Biological Co.) with a twofold enrichment of amino acids and 10% fetal calf serum (Grand Island Biological Co.) for 48 hr with the addition of an equal volume of medium at 24 hr. Under such conditions, the doubling time was 15–20 hr. Meth-A cells (1×10^6 cells/ml) were cultured in the previously mentioned medium for 18 hr. Doubling time was about 18 hr, and only one growth cycle occurred under these conditions. EL-4 cells (7.5×10^6 cells/ml) were sustained for the purpose of labeling for 15 hr in RPMI 1640 medium (Microbiological Associates, Inc.) containing 20 μg/ml of penicillin and streptomycin and 10% fetal calf serum. Cell counts usually remained unchanged during this time, but declined thereafter. L-[^{3}H]Fucose (4.3 Ci/mmole), D-[1-^{14}C]glucosamine (51.4 mCi/mmole), D-[6-^{3}H]glucosamine (1.3 Ci/mmole), D-[6-^{3}H]galactose (231 mCi/mmole), and D-[1-^{14}C]mannose (37.6 mCi/mmole) were purchased from New England Nuclear Corp. Radioactive sugars (usually 0.2–1 mCi) were added to the culture medium and, after incubation for a time previously determined to result in maximum label incorporation, cells were collected by centrifugation at 1000 rpm for 10 min and washed twice with 0.9% NaCl. About 10% of the labeled fucose, glucosamine, and galactose added to the medium was incorporated into the cells, while incorporation of mannose was slightly less effective (Table I).

Partial Purification of Carbohydrate-Labeled H-2 Alloantigens from Cultured Tumor Cells. The over-all procedures

[1] A preliminary report of this work is presented elsewhere (Muramatsu and Nathenson, 1970b).

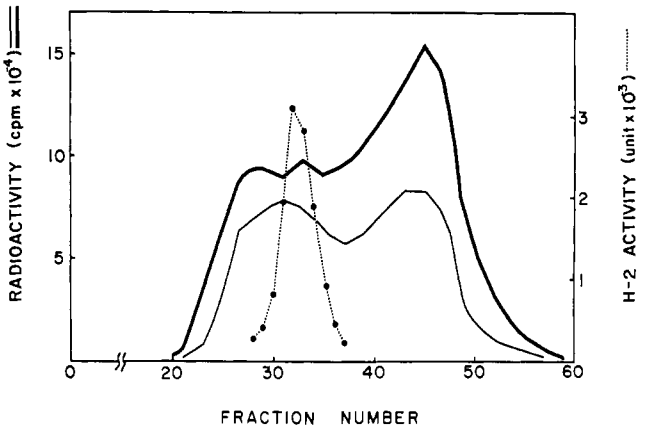

FIGURE 1: Sephadex G-150 column chromatography of papain-solubilized membrane glycoproteins from MTC cells labeled with [^{3}H]fucose. The [^{3}H]fucose-labeled material applied on the column contained 36.8 mg of papain-solubilized membrane protein from 3.54×10^9 cells, 2.4×10^4 units of *H-2* specificities 4, 10, 13 activity, and 3.55×10^6 cpm of radioactivity. The column (0.9×90 cm) was equilibrated and eluted with 0.15 M NaCl–0.01 M Tris-HCl (pH 8.4). The eluate was collected in 1.3-ml fractions and *H-2* antigenic activity for *H-2* specificities 4, 10, 13 (---●---) and radioactivity (heavy solid line) measured. For comparison the radiolabel profile of a similar preparation (1.96×10^6 cpm) labeled with [^{3}H]glucosamine (light solid line) is also shown. The void volume of the column was at fraction 19. Radioactivities and *H-2* activities are expressed as cpm or units per column fraction.

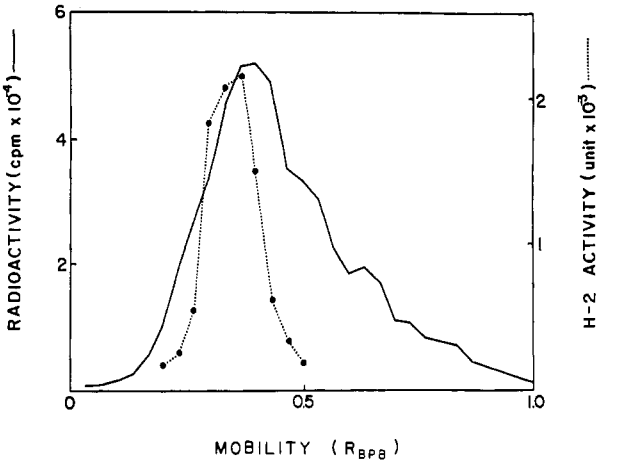

FIGURE 2: Disc gel electrophoresis step for the partial purification of [^{3}H]fucose-labeled *H-2* alloantigen from MTC cells. *H-2* alloantigen partially purified by Sephadex G-150 column chromatography as described in Figure 1 (1.2×10^4 units of *H-2* activity for specificities 4, 10, 13, 6.3×10^5 cpm of radioactivity, 0.99 mg of protein) was applied to the top of the spacer of a 7.5% polyacrylamide gel (0.9×15 cm) and electrophoresis was carried out as described previously (Shimada and Nathenson, 1969). After the run, gels were cut into 0.5-cm pieces and eluted with 0.5 ml of 0.15 M NaCl–0.01 M Tris-HCl (pH 8.4) three times, and counted (——) or assayed for activity (●---●).

are applications of the method of Shimada and Nathenson (1969) used for spleen cells. In three typical preparations the washed cells (approximately 4×10^9 of MTC cells and EL-4 cells, 2×10^9 of Meth-A cells) were suspended at 20 times their packed volume in 0.3% NaCl–0.01 M Tris-HCl (pH 8.4) buffer and homogenized with a Potter Teflon homogenizer for 3 min. After centrifugation at 2000 rpm for 10 min, the pellet was once more extracted with this buffer and, finally, with 0.1% NaCl–0.01 M Tris-HCl (pH 8.4) buffer. From the combined supernatant solutions, the crude membrane fraction was recovered by ultracentrifugation at 105,000*g* for 2 hr. These fractions had the following protein content and *H-2* activity: MTC cells, 90 mg of protein, 1.0×10^5 units of *H-2* activity for specificities 4, 10, 13; Meth-A cells, 150 mg of protein, 2.3×10^5 units of *H-2* activity for specificities 4, 10, 13; EL-4 cells, 80 mg of protein, 6.5×10^4 units of *H-2* activity for specificity 5. About 30–40% of the radioactive sugars incorporated into the cells were recovered in the membrane fraction (Table I).

The *H-2* alloantigens were solubilized by papain digestion (Shimada and Nathenson, 1969), using a 30-min incubation period for the MTC-cell membranes and 1 hr for the Meth-A and EL-4 membranes. This procedure solubilized 30–50% of the radioactive sugars from crude membranes and about 20% of the *H-2* alloantigenic activity. The supernatant of the papain digest was concentrated by ultrafiltration using a collodion bag and then subjected to Sephadex G-150 column chromatography (0.9×90 cm) in 0.01 M Tris-HCl (pH 8.4) buffer containing 0.15 M NaCl (*cf.* Figure 1). The *H-2* alloantigen fraction was used as the partially purified *H-2* alloantigen fraction, except in the experiments with the MTC cells, where the antigen was further purified by disc gel electrophoresis as described in Figure 2. The partially purified fraction contained, on the average, the following protein and *H-2* activity. For MTC cells, 0.4 mg of protein, 9×10^3 units of *H-2* activity for specificities 4, 10, 13; for Meth-A cells, 3.2 mg of protein, 3×10^4 units of *H-2* activity for specificities 4, 10, 13; and for EL-4 cells, 1.0 mg of protein, 8×10^3 units of *H-2* activity for specificity 5.

Purification of Carbohydrate-Labeled H-2 Alloantigen by Antigen–Antibody Complex Formation. The partially purified *H-2* alloantigens were complexed with antiserum and the complex was isolated from unreacted materials by the method described previously (Muramatsu and Nathenson, 1970a). An example of such a purification step is shown in Figure 3.

Identification of Radioactive Sugars. Descending paper chromatography was carried out on Whatman No. 3MM paper. The solvents used were: A, 1-butanol–pyridine–water (6:4:3, v/v) and B, 1-butanol–1-propanol–0.1 N HCl (1:2:1, v/v). For the identification of sugars in the [^{3}H]fucose experiments, radioactive materials were hydrolyzed by Dowex 50 (H^+) in 0.01 N H_2SO_4 at 100° for 15 hr. In the [^{3}H]galactose or [^{14}C]mannose experiments, radioactive materials were hydrolyzed by 1 N H_2SO_4 at 100° for 15 hr and desalted on columns of Dowex 1-X8 (CO_3^{2-}) and Dowex 50 (H^+). In [^{3}H]glucosamine or [^{14}C]glucosamine experiments, the radioactive materials were hydrolyzed with 4 N HCl at 100° *in vacuo* for 4 hr, and HCl was removed by lyophilization.

The immunologically purified radiolabeled *H-2* alloantigen was first dialyzed against distilled water and after hydrolysis the preparation subjected to paper chromatography in solvent system A. The dried chromatograms were cut into 0.5-cm strips and directly counted by a liquid scintillation counter.

Radioactive glucosamine was also identified by column chromatography using a Beckman Model 120C amino acid analyzer. The eluate from the column was collected before color development and counted.

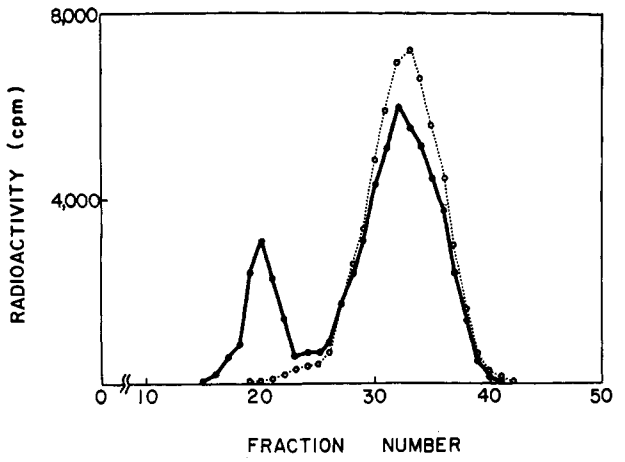

FIGURE 3: Isolation of antigen–antibody complex by Sephadex G-150 column chromatography. [^{3}H]Fucose-labeled partially purified *H-2* alloantigen (7.0×10^4 cpm) from EL-4 (*H-2*^{b}) was treated with 11.2 mg of IgG fraction from antiserum anti-*H-2*^{d} and chromatographed (●). As the control run, the same amount of antigen was mixed with an IgG fraction (13.7 mg) from antiserum anti-*H-2*^{d} and chromatographed (○). The reaction mixtures were chromatographed on a column of Sephadex G-150 (0.9×90 cm) and equilibrated and eluted with 0.15 M NaCl–0.01 M Tris-HCl (pH 8.4); 1.3-ml fractions were collected. The void volume was at fraction 19.

SCHEME I: Preparation of Radiolabeled Glycopeptides from *H-2* Alloantigens.

A. Radiolabeled cells
   1. Cell lysis and differential centrifugation

↓

B. Crude membrane fraction
   1. Papain digestion
   2. Sephadex G-150 column chromatography
   (3. Disc gel electrophoresis)

↓

C. Partially purified radiolabeled *H-2* alloantigen
   1. Formation of radiolabeled antigen–antibody complex
   2. Isolation of radiolabeled antigen–antibody complex by Sephadex G-150 chromatography

↓

D. Purified radiolabeled *H-2* alloantigen
   1. Pronase digestion

↓

E. Radiolabeled glycopeptides

For the identification of sialic acids, the glucosamine-labeled materials were hydrolyzed with 0.1 N H_2SO_4 for 1 hr at 80°. The hydrolysate was applied to a column of Dowex 1-X8 (formate form). Sialic acids were eluted by 0.3 N formic acid. For the further identification of sialic acids, formic acid was removed by ether extraction, and the eluate was chromatographed in solvent system B.

Pronase Digestion of Carbohydrate-Labeled H-2 Alloantigens Complexed with Alloantibody. The *H-2* alloantigen–antibody complex (protein content, including that of IgG, 100 μg or less) was hydrolyzed with 5 mg of pronase (Sigma Chemical Co.) in 1 ml of 0.1 M Tris-HCl (pH 8.4) containing 0.01 M $CaCl_2$ and 0.135 M NaCl at 37° with a small amount of toluene. After 24 hr an additional 5 mg of pronase was added and incubation was continued for another 24 hr. The overall method for the preparation of *H-2* glycopeptides is outlined on a flow sheet (Scheme I).

Sephadex G-50 Column Chromatography of Glycopeptides. The glycopeptide solution (about 0.5 ml) was applied to a column of Sephadex G-50 fine (0.9×110 cm) equilibrated and eluted with 0.05 M NaCl–0.0033 M Tris-HCl (pH 8.4) buffer, and 1.3-ml fractions were collected except in the double labeling experiment where 0.65-ml fractions were collected.

DEAE-Sephadex A-25 Column Chromatography of Glycopeptides. The glycopeptide solution was desalted on a column of Sephadex G-15 (0.9×50 cm) equilibrated with 0.01 M Tris-HCl (pH 8.4) and then applied to a column of DEAE-Sephadex (0.9×10 cm) equilibrated with 0.01 M Tris-HCl (pH 8.4). Elution was begun with 15 ml of the same buffer, and then a linear gradient elution was initiated using a two-chamber system with mixing of the initial and limit eluents in the mixing chamber. The limit chamber contained 100 ml of 0.3 M NaCl–0.01 M Tris-HCl (pH 8.4) and the mixing chamber, 100 ml of 0.01 M Tris-HCl (pH 8.4). Two milliliters per fraction were collected.

Other Chemicals and Methods. Thyroglobulin glycopeptide (unit B) was a kind gift of Drs. T. Arima and R. G. Spiro. Ovalbumin glycopeptide and fetuin glycopeptide were prepared by the method of Yamashina and Makino (1962) and by the method of Spiro (1962), respectively. Protein content was measured by the method of Lowry *et al.* (1951) using crystalline bovine plasma albumin as a standard. Radioactivity was determined by a Beckman Model LS-250 liquid scintillation counter using a toluene Omnifluor (Beckman Instruments) (4 g/l. of toluene) and Triton X-100 (0.43 l./l. of toluene) scintillation mixture; 1 ml or less of sample was added to 10 ml of counting mixture.

Results

Purification of the Papain Solubilized H-2 Alloantigen Fragments Labeled with [^{14}C]- and [^{3}H]Monosaccharides. PAPAIN-SOLUBILIZED MEMBRANE GLYCOPROTEINS. Properties and partial fractionation. The papain-solubilized membrane glycoproteins labeled with radioactive sugars were extremely heterogeneous with respect to size and charge. An example of such a size range can be seen from the pattern of radiolabeled material during Sephadex G-150 column chromatography of a crude papain supernatant (Figure 1). Only a small portion of this digest material contained *H-2* alloantigenic activity. When the *H-2* active material was pooled, and subjected to disc gel electrophoresis, some heterogeneity still remained (Figure 2), as seen by the broad spread of radiolabeled material; however, considerable purification had been effected by these two steps and the material at the latter stage was about 40% pure, as judged by the amount of radiolabel which would complex with antibody.

SPECIFIC IMMUNOLOGICAL REACTION AS A FINAL PURIFICATION STEP. Previous studies using mouse spleen cells as source have shown that purification of *H-2* alloantigen fragments required a series of rather laborious fractionation steps including Sephadex G-150, CM-Sephadex, DEAE-Sephadex chromatography, and disc gel electrophoresis. In a preliminary study, we described an alternate method for purifying radio-

TABLE II: Specificity of Radiolabeled Antigen–Antibody Complex Formation.[a]

Type of Radiolabeled Alloantigen	Antiserum or IgG Fraction	*H-2* Alloantigen Preparation Used As Inhibitor of Complex Formation	% Complex Formation
A. Partially purified *H-2*^{b} alloantigen from EL-4 ([^{3}H]fucose label, 95 units of H-2.5 activity, 12.2 μg)	(1) Antiserum against *H-2*^{b} specificities 2, 5, 22, 33 (1940 μg)	None	17.8
	(2) Antiserum against *H-2*^{d} specificities 3, 4, 8, 10, 13, 31 (1850 μg)	None	1.4
	(3) IgG fraction against *H-2*^{b} specificities 2, 5, 22, 33 (318 μg)	None	16.5
	(4) Same as above	*H-2*^{b} alloantigen from C57BL/6 mice (830 units of H-2.5 activity, 18.0 μg)	2.1
	(5) IgG fraction against *H-2*^{d} specificities 3, 4, 8, 10, 13, 31 (348 μg)	None	1.8
	(6) None	None	1.4
B. Partially purified *H-2*^{d} alloantigen from Meth-A ([^{3}H]fucose label, 91 units of H-2.4, 10, 13 activity, 9.1 μg)	(1) IgG fraction against *H-2*^{d} specificities 3, 4, 8, 10, 13, 31 (249 μg)	None	19.3
	(2) Same as above	*H-2*^{d} alloantigen from DBA/2 mice (2670 units of H-2.4, 10, 13 activity, 66.7 μg)	3.8
	(3) IgG fraction against *H-2*^{d} specificities 2, 5, 22, 33 (273 μg)	None	2.4
	(4) None	None	2.1

[a] Experimental procedures were described previously (Muramatsu and Nathenson, 1970a).

labeled *H-2* alloantigen directly, using *H-2* alloantibody (Cullen and Nathenson, 1969; Muramatsu and Nathenson, 1970a). By this method, *H-2* alloantigens at various stages of fractionation could be complexed with anti-*H-2* alloantibody and the soluble antigen–antibody complex then isolated from unreactive materials by gel filtration or discontinuous electrophoresis on polyacrylamide gels.

To establish the specificity of this purification step, we presented preliminary data with material from one strain, the MTC cell (Muramatsu and Nathenson, 1970a). More extensive data using disc electrophoresis on polyacrylamide gels is presented in Table II which characterizes the properties of this immune reaction and establishes the validity for its use to isolate the radiolabeled product for the studies described here. These experiments demonstrate that: (1) partially purified carbohydrate-labeled *H-2* alloantigen from the *H-2*^{b} strain formed a complex with antiserum or an IgG fraction of the antiserum directed against *H-2*^{b} specificities 2, 5, 22, 33 but not with an antiserum or IgG fraction against *H-2*^{d} specificities 3, 4, 8, 10, 13, 31, and *vice versa;* (2) the IgG fraction purified from alloantiserum was as effective as unfractionated antiserum; (3) preincubation of alloantibody with an excess of highly purified *H-2* alloantigens (not radioactively labeled) from spleens of the same *H-2* genotype completely inhibited the complex formation.

For large-scale preparations of purified materials the radiolabeled antigen–antibody reaction products were separated from the unreactive materials by gel filtration (Cullen and Nathenson, 1969). IgG fractions from *H-2* alloantisera used for these preparations were either anti-*H-2*^{b} (specificities 2, 5, 22, 33) or anti-*H-2*^{d} (specificities 3, 4, 8, 10, 13, 31). One of the typical results is illustrated in Figure 3. An aliquot of [^{3}H]fucose-labeled partially purified *H-2* alloantigen from EL-4 cells (*H-2*^{b}) was reacted with the IgG fraction from an antiserum against *H-2*^{b} specificities and the mixture then applied to a Sephadex G-150 column. The antigen–antibody complex appeared in the void volume as a sharp peak. We pooled the material in the fractions 17–21 (9.25×10^3 cpm) as purified *H-2* alloantigen complexed with antibody. As a control experiment, the partially purified antigen was mixed with an aliquot of an IgG fraction from antiserum against *H-2*^{d} specificities and the mixture applied on the column. No significant radioactivity (138 cpm) appeared in the complex region. Therefore, we could estimate the purity of the labeled antigen as about 98%. Purities of more than 95% were usual.

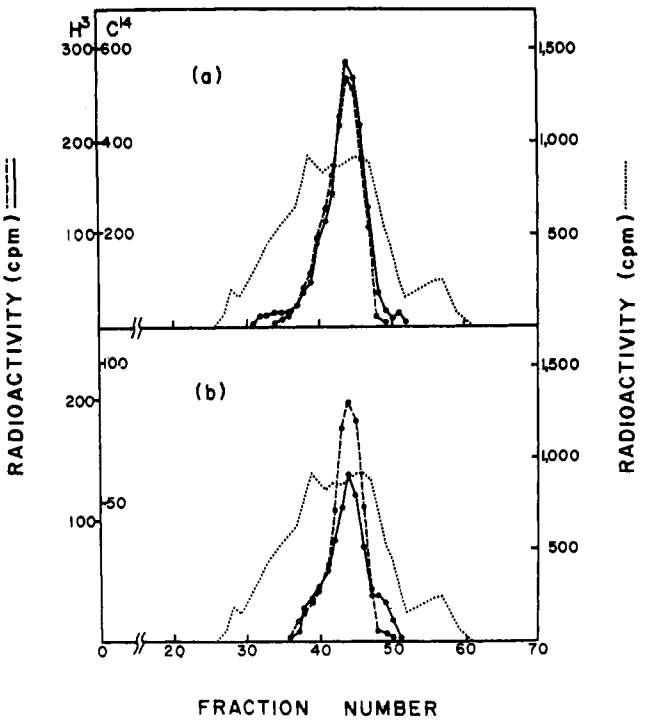

FIGURE 4: Sephadex G-50 column chromatography of double-labeled glycopeptides from EL-4 cells: (a) profile of [³H]fucose- (1750 cpm added, ●) and [¹⁴C]glucosamine- (3450 cpm added, ○) labeled glycopeptides. A profile for an [³H]fucose-labeled crude membrane glycopeptide preparation from a separate experiment is plotted in the same graph for comparison (17,900 cpm added, ----); (b) profile of [³H]galactose-labeled (1000 cpm added, ○) and [¹⁴C]mannose- (370 cpm, ●) double-labeled *H-2* glycopeptides. The same crude membrane glycopeptide profile (----) as used for (a) is also plotted. The crude membrane glycopeptides were prepared by pronase digestion of the radiolabeled crude membranes under the same conditions used for *H-2* alloantigens. Chromatography performed as described in Materials and Methods. The void volume was at fraction 28.

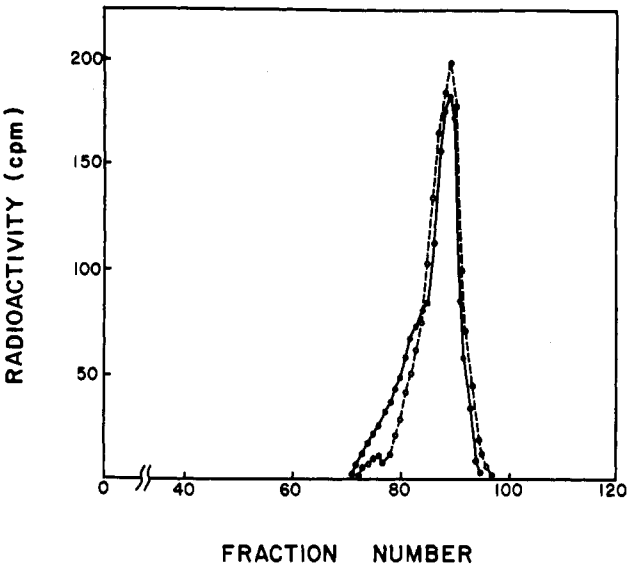

FIGURE 5: Comparison of Sephadex G-50 column chromatographic patterns of *H-2* glycopeptides from Meth-A cells and EL-4 cells by a double-label mixture experiment. [³H]Fucose-labeled *H-2* glycopeptides from Meth-A cells (1600 cpm added, ●) and [¹⁴C]glucosamine-labeled *H-2* glycopeptides from EL-4 cells (1550 cpm, ○) were mixed before chromatography which was carried out as described in Materials and Methods. The void volume was at tube 56.

Identification of Radioactive Sugars Incorporated into Purified H-2 Alloantigens. Radioactive materials incorporated into immunologically purified *H-2* alloantigens were identified after acid hydrolysis of the purified antigens. In the [³H]fucose experiment, [³H]galactose experiment, and [¹⁴C]mannose experiment, more than 90% of radioactivity on the paper chromatogram coincided with the sugars originally added in radiolabeled form. In [³H]- or [¹⁴C]glucosamine-labeling experiments, only one radioactive spot corresponding to glucosamine was detected by paper chromatography after hydrolysis by 4 N HCl for 4 hr. The identity of the ³H-labeled material in the hydrolysate was confirmed in the case of the MTC-cell alloantigens by column chromatography using the amino acid analyzer where ³H label appeared only in the glucosamine region. Since no radiolabel was found in the galactosamine region of the chromatogram, the absence of galactosamine was confirmed. A significant amount of radioactivity was also recovered as sialic acid after mild acid hydrolysis of the alloantigen and Dowex 1-X8 (formate form) column chromatography. The per cent of radioactivity recovered as sialic acid with respect to the total radioactivity in the purified antigens was as follows: 15% (*H-2* alloantigen from MTC cells), 16% (that from Meth-A cells), and 25% (that from EL-4 cells). The major portion (70%) of sialic acid in the purified antigen from MTC cells coincided with *N*-glycolylneuraminic acid, and the remainder (30%) to *N*-acetylneuraminic acid upon paper chromatography.

These data show that the immunologically purified *H-2* alloantigens from tumor cells of both $H\text{-}2^b$ and $H\text{-}2^d$ strains have fucose, mannose, galactose, glucosamine, and sialic acid as integral components.

Analysis of the Glycopeptides from H-2 Alloantigens and from Crude Membrane Fractions by Sephadex G-50 Column Chromatography. We prepared radioactive glycopeptides of *H-2* alloantigens by pronase digestion of the immunologically purified carbohydrate-labeled alloantigens, and analyzed the glycopeptides by Sephadex G-50 column chromatography. The profiles of the purified *H-2* glycopeptides from EL-4 cells ($H\text{-}2^b$) are illustrated in Figure 4. The glycopeptides had been doubly labeled with [³H]fucose and [¹⁴C]glucosamine in one experiment (Figure 4a) with [³H]galactose and [¹⁴C]-mannose in the other experiment (Figure 4b). The radioactivity pattern for each of the four monosaccharides showed a sharp peak at the identical fraction number (tube 44). Thus all four monosaccharides reside on glycopeptides of the same molecular weight.

In a comparison of the glycopeptides from alloantigens of different sources, labeled *H-2* glycopeptides from MTC ($H\text{-}2^d$) and Meth-A ($H\text{-}2^d$) were also tested on the Sephadex G-50 column used for Figure 4. The [³H]fucose-labeled glycopeptides from MTC cell alloantigen (Muramatsu and Nathenson, 1970a) and Meth-A alloantigen (data not shown) both formed a sharp peak at fraction 44. Thus the elution volumes appeared identical for the glycopeptides from all the *H-2* alloantigen glycoproteins of three different types of tumor cells, two of identical *H-2* genotype, and the third a different genotype.

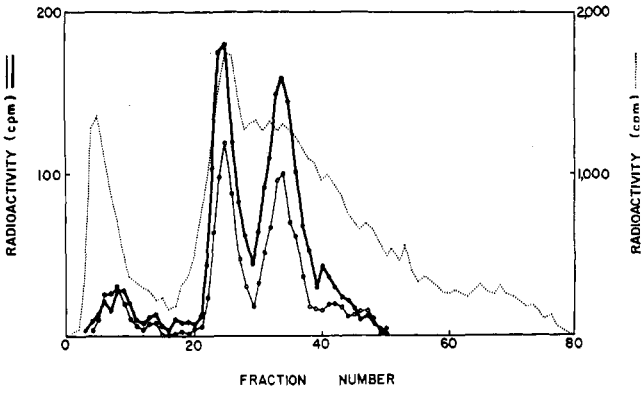

FIGURE 6: DEAE-Sephadex column chromatography of *H-2* glycopeptides from *H-2* glycoprotein of EL-4 cells doubly labeled with [^{14}C]glucosamine (2200 cpm applied, ●) and [^{3}H]fucose (1350 cpm applied, ○). The profile for [^{3}H]fucose-labeled crude membrane glycopeptides (71,000 cpm, ---) is also shown. Chromatography was performed as described in Materials and Methods.

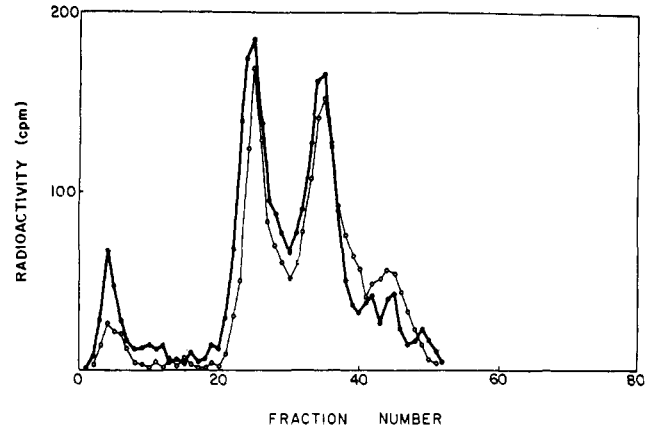

FIGURE 7: Comparison of *H-2* glycopeptides from Meth-A cells and EL-4 cells using DEAE-Sephadex column chromatography. [^{3}H]Fucose-labeled *H-2* glycopeptides from Meth-A cells (2300 cpm, ○) were mixed with [^{14}C]glucosamine-labeled *H-2* glycopeptides from EL-4 cells (2700 cpm, ●) and chromatographed as described in Materials and Methods.

During the Sephadex G-50 column chromatography, a one-tube difference in the region of fraction 44 would correspond to the difference of molecular weight of about 400. Therefore, size differences of one or possibly two sugar residues between the *H-2* glycopeptides from different tumor cells would be undetectable. In a double-labeling experiment to examine the elution profile with finer resolution *H-2* glycopeptides from *H-2* alloantigens from EL-4 cells (*H-2^{b}*) and Meth-A cells (*H-2^{d}*) were chosen for comparison, for they are different in both cell type (lymphoma *vs.* fibrosarcoma) and *H-2* specificity (*H-2^{b} vs. H-2^{d}*). For this experiment, the volume of each fraction was reduced by two. After confirming the coincidence of the [^{3}H]fucose peak and [^{14}C]glucosamine peak for the *H-2* glycopeptides from EL-4 cells, a mixture of [^{14}C]-glucosamine-labeled *H-2* glycopeptides from EL-4 cells and of [^{3}H]fucose-labeled *H-2* glycopeptides from Meth-A cells was analyzed. As shown in Figure 5, the ^{3}H and ^{14}C peaks coincided, although some very small differences were found in fractions 75–84. This result clearly indicates that, by the gel filtration criterion, the molecular weights of the major *H-2* glycopeptides from EL-4 cells and Meth-A cells are identical within the error of less than one carbohydrate residue.

In view of the uniquely sized glycopeptides of *H-2* alloantigen we examined the properties of the glycopeptides of crude membranes as well, and the dash lines in Figure 4a,b shows the pattern obtained. The extremely heterogeneous nature of the crude membrane glycopeptides is seen in contrast to the high degree of homogeneity of *H-2* glycopeptides with respect to size.

Since the crude membrane preparation contained various kinds of membranes and particles, while the *H-2* glycopeptides were mainly derived from the cell surface, we also compared the glycopeptide pattern of papain-solubilized material from the cell surface alone. B. D. Schwartz and S. G. Nathenson (in preparation) had found that crude papain digestion of Meth-A tumor cells releases a major portion of *H-2* alloantigenic activity, without lysis of the cells. We treated the [^{3}H]fucose-labeled Meth-A cells by crude papain according to this method and found that the cell surface glycopeptides prepared from the solubilized materials by pronase digestion were also heterogeneous with respect to size, and had a Sephadex pattern very similar to that seen in Figure 4 for crude membranes.

In order to obtain information on the molecular weight of *H-2* glycopeptides, we calibrated the Sephadex G-50 column using three glycopeptides as standards. The peak of the fetuin glycopeptide (average molecular weight $\sim$ 4400) was at fraction 41, that of thyroglobulin glycopeptide unit B (average molecular weight $\sim$ 4100) at fraction 42, that of ovalbumin glycopeptide (average molecular weight $\sim$ 1550) at fraction 51. We estimated that the average molecular weight of the major glycopeptides from three types of tumor cells was 3300 $\pm$ 500 by the method of Andrews (Andrews, 1964; Bhatti and Clamp, 1968).

DEAE-Sephadex Column Chromatography of Glycopeptides from H-2 Alloantigens. We examined the charge of *H-2* glycopeptides by DEAE-Sephadex A-25 column chromatography. *H-2* glycopeptides from EL-4 cells doubly labeled with [^{3}H]-fucose and [^{14}C]glucosamine revealed two major sharp peaks (Figure 6) with two minor radioactive fractions, as compared to the pattern from another chromatographic run of glycopeptides from crude membranes which showed a heterogeneous smear of materials. *H-2* glycopeptides from Meth-A cells labeled with [^{3}H]fucose also revealed two major sharp peaks and two minor radioactive fractions, whereas whole membrane glycopeptides were highly heterogeneous (data not shown). These results clearly indicate that *H-2* glycopeptides are mainly composed of two types of glycopeptides that differ in charge but are almost identical in size.

To compare more accurately the *H-2* glycopeptides from EL-4 cells and those from Meth-A cells, we carried out a double-labeling mixture experiment using DEAE-Sephadex column chromatography. The patterns of radioactivity were essentially identical upon chromatography of the mixture of [^{14}C]glucosamine-labeled *H-2* glycopeptides from EL-4 cells and [^{3}H]fucose-labeled *H-2* glycopeptides from Meth-A cells (Figure 7). This result confirms the close similarity of the *H-2^{b}* and *H-2^{d}* glycopeptides from the two types of tumor cells with differing *H-2* specificities.

Discussion

Glycoproteins, as integral components of animal membrane systems (Kathan and Winzler, 1963; Yamashina *et al.*, 1964; Brunngraber and Brown, 1967; Molnar, 1967; Miyazima *et al.*, 1969; Pepper and Jamieson, 1969), are of great interest because of their probable role in specific functions of the cellular surface such as cell association, contact inhibition, recognition of foreign or tumor cells, etc. (Gesner and Ginsburg, 1964; Cox and Gesner, 1965; Humphreys, 1967; Meezan *et al.*, 1969; Oppenheimer *et al.*, 1969). Relatively little is known about the biochemical nature of the carbohydrate moieties of most membrane specific glycoproteins. Studies have been mainly restricted to those from red cell membranes: *i.e.*, the MN-blood group substances (Winzler, 1969; Thomas and Winzler, 1969), the influenza virus receptor (Winzler, 1969; Kathan and Winzler, 1963), the phytohaemaglutinin receptor (Kornfeld and Kornfeld, 1969); and to those of specialized membranes such as the renal antigen from glomerular basement membrane (Shibata *et al.*, 1969), and the pigment protein from the rod outer segment disc system (Heller and Lawrence, 1970).

In other cells and tissues, glycopeptides prepared by protease digestion of unfractionated glycoproteins from membranes have been examined (Brunngraber and Brown, 1967; Meezan *et al.*, 1969). The membrane glycopeptides thus prepared were in most cases heterogeneous with respect to size or charge and were difficult to separate cleanly from each other.

In this paper, we established the general properties of the carbohydrate moieties of a single type of membrane located glycoprotein, the *H-2* alloantigen, which provides a convenient material for study because it can be assessed precisely by a specific immune detection system, has a clearly defined genetic control system (*cf.* Snell and Stimpfling, 1966), and is expressed in both normal cells and tumor cells. The tumor cells are especially suitable for isotope-labeling experiments.

Our results, together with the previous reports (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970a; Muramatsu and Nathenson, 1970a), have firmly established that *H-2* alloantigens are typical glycoproteins. The possibility of a glycolipid binding to a simple protein seems unlikely, because the carbohydrate-labeled *H-2* alloantigens lost no radioactivity when extracted with chloroform–methanol (unpublished observations).

As expressed in Table I our data show that carbohydrate moieties of *H-2* alloantigens represent only a small part of the total membrane-located carbohydrate of the tumor cells. About 4–6% of [^{3}H]fucose incorporated into crude membranes exists in the *H-2* alloantigen from tumor cells, while a much smaller per cent of membrane [^{3}H]galactose, [^{14}C]mannose, [^{3}H,^{14}C]glucosamine exists in the alloantigen. The finding that the *H-2* glycoprotein is a distinct entity which comprises only a small amount of the membrane material fits with recent studies which suggest that rather than being comprised of a single or a small number of protein subunits, animal cell membranes in reality consist of a rather large array of proteins and glycoproteins of widely different molecular weight (Kiehn and Holland, 1968, 1970; Rosenberg and Guidotti, 1969; Lenard, 1970; Meezan *et al.*, 1969).

In the present studies we identified galactose, mannose, fucose, glucosamine, and sialic acid as monosaccharide components of *H-2* alloantigens from tumor cells of both *H-2*^{b} and *H-2*^{d} strains. In the acid hydrolysate of the *H-2* alloantigens purified from spleen cells, mannose, galactose, and a small amount of fucose have been detected by thin-layer chromatography and color development by anisaldehyde–H_2SO_4 reagent (T. Muramatsu, A. Shimada, and S. G. Nathenson, unpublished observation). Therefore, we may conclude that the major neutral sugars are restricted to the three sugars. The absence of galactosamine in *H-2* alloantigens purified from spleen cells and tumor cells has been demonstrated by column chromatography using an amino acid analyzer (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970a,b). Thus, the overall carbohydrate composition of *H-2* alloantigens appears similar to that of the serum glycoproteins. The phytohaemagglutinin receptor from red cell membranes (Kornfeld and Kornfeld, 1969), glycopeptides from platelet membranes (Pepper and Jamieson, 1969), and glycopeptides from Sindbis virus membranes (Burge and Strauss, 1970) also have the serum glycoprotein-type carbohydrate composition.

The molecular weight of the papain-solubilized *H-2* alloantigen glycoprotein (class I) from Meth-A cells or EL-4 cells is about 57,000 of which 10% is carbohydrate (Yamane and Nathenson, 1970b). We estimate that the molecular weight of the *H-2* glycopeptides is approximately 3300 and we would expect that due to the severe conditions for pronase digestion very few amino acids are still present in these glycopeptides. Therefore, we propose that *H-2* alloantigen fragments solubilized by papain would have two carbohydrate chains per molecule. Of course, it is possible that pronase might not split a peptide bond linkage between two carbohydrate chains attached to adjacent or close amino acids, and hence each glycopeptide isolated might consist of two or more chains. Our findings that all radiolabeled sugars examined have identical patterns when their glycopeptides are chromatographed by Sephadex G-50 or DEAE-Sephadex suggest that all components are present in every glycopeptide chain.

Since all our studies reported herein were carried out with tumor cells it would be possible that the carbohydrate of the antigen of normal tissues might have different properties. We have recently carried out preliminary studies on normal spleen cells and found that at least with respect to Sephadex G-50 behavior the *H-2* glycopeptides are identical with those from tumor cells (T. Muramatsu and S. G. Nathenson, unpublished observations).

It is not yet clear whether the carbohydrate moieties are related to the immunological specificities of the antigen or to some other physiological function. At the present time it appears reasonable to consider that the *H-2* antigenic specificities are determined either wholly or in part by the primary protein structure. A few but reproducible differences in protein structure have been detected between *H-2* alloantigens from *H-2*^{b} strain and *H-2*^{d} strain by using thin-layer peptide mapping techniques (Shimada *et al.*, 1970). Our present finding of the close similarity of *H-2* glycopeptides from tumor cells of both *H-2*^{b} and *H-2*^{d} strain provides some indirect support for the above hypothesis. However, it is still quite possible that the similar glycopeptides are different in the order, configuration, and linkage of the sugar units, and that such fine structural architecture may relate to the immunological specificity of the antigens. In order to answer the questions clearly, other approaches are now in progress,

which include the removal of sugars in the antigens by purified glycosidases and detailed structural analysis of the glycopeptides. Sialic acid has already been removed from the antigens without any loss of the *H-2* alloantigenic activity (A. Shimada and S. G. Nathenson, submitted for publication), and the majority of the galactose moieties have also been removed by β-galactosidase of *Diplococcus pneumoniae* without changing the activity (T. Muramatsu and S. G. Nathenson, unpublished observation).

With regard to the biological relevance of the carbohydrate structure of membrane glycoproteins, the possibility arises that the carbohydrate chains are unique for every individual glycoprotein. Our findings would fit with this view. If so, however, the question of the control of such structures becomes important. Is this due to an array of different glycosyl transferases of different specificities? Or does the primary structure of the protein influence the carbohydrate structures? These and other questions will be of interest as other glycoproteins of mammalian cell surfaces are studied, and it is determined whether each glycoprotein in fact carries a unique carbohydrate chain. With this in mind, we have initiated studies on several other cell surface antigens with the hope of bringing more information to this problem.

Acknowledgment

We thank Dr. E. J. Hehre for his helpful criticism of this manuscript.

References

Andrews, P. (1964), *Biochem. J. 91*, 222.
Bhatti, T., and Clamp, J. R. (1968), *Biochim. Biophys. Acta 170*, 206.
Brunngraber, E. G., and Brown, B. D. (1967), *Biochem. J. 103*, 65.
Burge, B. W., and Strauss, J. H. (1970), *J. Mol. Biol. 47*, 449.
Cox, R. P., and Gesner, B. M. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 1571.
Cullen, S., and Nathenson, S. G. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 379.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Gesner, B. M., and Ginsburg, V. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 750.
Heller, J., and Lawrence, M. (1970), *Biochemistry 9*, 864.
Humphreys, T. (1967), *in* The Specificity of Cell Surfaces, Davis, B. D., and Warren, L., Ed., Englewood Cliffs, N. J., Prentice-Hall, p 195.
Kathan, R. H., and Winzler, R. J. (1963), *J. Biol. Chem. 238*, 21.
Kiehn, E. D., and Holland, J. J. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1370.
Kiehn, E. D., and Holland, J. J. (1970), *Biochemistry 9*, 1716.
Kornfeld, S., and Kornfeld, R. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 1439.
Lenard, J. (1970), *Biochemistry 9*, 1129.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Meezan, E., Wu, H. C., Black, P. H., and Robbins, P. W. (1969), *Biochemistry 8*, 2518.
Miyazima, N., Tomikawa, M., Kawasaki, T., and Yamashina, I. (1969), *J. Biochem. (Tokyo) 66*, 711.
Molnar, J. (1967), *Biochemistry 6*, 3064.
Muramatsu, T., and Nathenson, S. G. (1970a), *Biochem. Biophys. Res. Commun. 38*, 1.
Muramatsu, T., and Nathenson, S. G. (1970b), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 910.
Nathenson, S. G., and Davies, D. A. L. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 476.
Oppenheimer, S. B., Edidin, M., Orr, C. W., and Roseman, S. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 1395.
Pepper, D. S., and Jamieson, G. A. (1969), *Biochemistry 8*, 3362.
Rosenberg, S. A., and Guidotti, G. (1969), *J. Biol. Chem. 244*, 5118.
Sanderson, A. R. (1964), *Brit. J. Exp. Pathol. 45*, 398.
Shibata, S., Miyakawa, Y., Naruse, T., Nagasawa, T., and Takuma, T. (1969), *J. Immunol. 102*, 593.
Shimada, A., and Nathenson, S. G. (1969), *Biochemistry 8*, 4048.
Shimada, A., Yamane, K., and Nathenson, S. G. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 691.
Snell, G. D., and Stimpfling, J. H. (1966), *in* Biology of the Laboratory Mouse, Green, E., Ed., New York, N. Y., McGraw-Hill, p 457.
Spiro, R. G. (1962), *J. Biol. Chem. 237*, 382.
Thomas, D. B., and Winzler, R. J. (1969), *J. Biol. Chem. 244*, 5943.
Wigzell, H. (1965), *Transplantation 3*, 423.
Winzler, R. J. (1969), *in* Cellular Recognition, Smith, R. T., and Good, R. A., Ed., New York, N. Y., Appleton-Century-Crofts, p 11.
Yamane, K., and Nathenson, S. G. (1970a), *Biochemistry 9*, 1336.
Yamane, K., and Nathenson, S. G. (1970b), *Biochemistry 9* (in press).
Yamashina, I., Izumi, K., and Naka, H. (1964), *J. Biochem. (Tokyo) 55*, 652.
Yamashina, I., and Makino, M. (1962), *J. Biochem. (Tokyo) 51*, 359.