Comparative Studies on the Carbohydrate-Containing Membrane Components of Normal and Virus-Transformed Mouse Fibroblasts. I. Glucosamine-Labeling Patterns in 3T3, Spontaneously Transformed 3T3, and SV-40-Transformed 3T3 Cells*

Henry C. Wu,† Elias Meezan,‡ Paul H. Black, and Phillips W. Robbins

ABSTRACT: In a double-labeling experiment to detect any change in the composition of glycoproteins and glycolipids following oncogenic virus transformation of mouse fibroblasts, [^{3}H]- and [^{14}C]glucosamine were used to label cell line 3T3 and SV-40 virus-transformed 3T3 cells. After growing separately in the presence of radioactive glucosamine, 3T3 and SV-40-3T3 cells were harvested and mixed in the following combinations: [^{3}H]SV-40-3T3 and [^{14}C]3T3 (mixture I), and [^{3}H]3T3 and [^{14}C]SV-40-3T3 (mixture II). Both mixtures were homogenized and separated into subcellular fractions by the method of Wallach. The ^{3}H to ^{14}C ratios were also determined for sialic acid, galactosamine, and glucosamine isolated from each fraction. Analysis of the relative composition of membrane glycoproteins and glycolipids in 3T3 and SV-40-3T3 showed a much lower sialic acid and galactosamine content and a reciprocal increase in the relative content of glucosamine in SV-40-3T3 as compared with 3T3. These differences in the relative composition of amino sugars between 3T3 and SV-40-3T3 were seen in all particulate fractions but not in the nucleotide sugar fraction. Thus the changes in the membrane carbohydrate composition in virus-transformed cells could not be attributed to a lack of particular nucleotide sugar precursors. Measurements of the absolute amounts of neutral and amino sugars present in the particulate fractions of 3T3 and SV-40-3T3 by the isotope dilution technique of Ginsburg revealed a marked decrease in both neutral and amino sugars per milligram of protein in SV-40-3T3 as compared with 3T3. Although the double-labeling technique had revealed minor differences in the relative composition of membrane carbohydrates between 3T3 and spontaneously transformed 3T3, measurements of sugar contents in spontaneously transformed 3T3 by the isotope dilution technique nevertheless showed decreased amounts of both neutral and amino sugars with the levels of sugar per milligram of protein intermediate between those present in 3T3 and SV-40-3T3. The implications of these changes in membrane glycoproteins and glycolipids following viral and spontaneous transformation are discussed.

The malignant transformed cell is characterized by alterations of the cell surface presumably leading to a loss of contact inhibition. It is possible that these changes in the cell surface are responsible directly or indirectly for the loss of the regulatory restraint on DNA synthesis which characterizes the normal contact-inhibited cell. Thus, cell surface changes might give some insight into the events leading to neoplasia.

It seemed important, therefore, to determine whether any biochemical differences in surface membrane components could be disclosed between normal and virus-transformed cells. Antigenic changes have previously been dmeonstrated since virus-transformed cells have new, specific antigens on the cell surface, and these virus-specific antigens are believed to be responsible for the rejection of transplanted cells to a previously immunized animal (Habel, 1961; Sjögren *et al.*, 1961). Other differences between normal and transformed cells in electrostatic charge (Forrester *et al.*, 1962, 1964), and acid mucopolysaccharides (Defendi and Gasic, 1963) determined by electrophoretic mobility studies and histochemical techniques have been reported. Biochemical methodology now available might be used to investigate these problems in more detail.

Studies to compare the virus-transformed cell line with the untransformed line have made use of a double-labeling technique. Glucosamine was chosen as the labeled compound because it is the precursor of sialic acid, *N*-acetylglucosamine and *N*-acetylgalactosamine, all of which are likely constituents of cell surface material (Kornfeld and Ginsburg, 1966). In addition, glucosamine would be expected to be relatively specific in labeling predominantly only those three compounds. The present investigations report the results of biochemical studies of the carbohydrate-containing membrane components of normal and SV40-transformed cells.

Experimental Procedures

Materials. The nitrogen pressure homogenizer was obtained

* From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts and Massachusetts General Hospital, Boston, Massachusetts. *Received December 23, 1968.* This investigation was supported by U. S. Public Health Service Grant AM 06803 from the National Institutes of Health to P. W. R. and by U. S. Public Health Service Research Grant CA 10126 from the National Cancer Institute to P. H. B.

† Fellow of the Helen Hay Whitney Foundation. Present address: Division of Biology, California Institute of Technology, Pasadena, Calif.

‡ Fellow of the Jane Coffin Childs Fund for Medical Research (1966–1967) and Fellow of the Medical Foundation, Boston, Mass. (1967–1969).

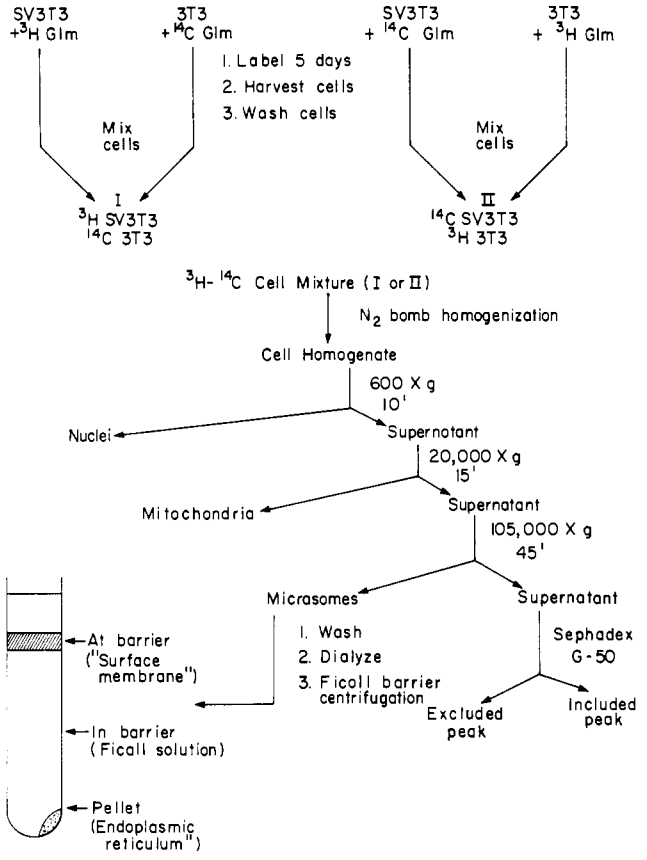

FIGURE 1: Glucosamine double labeling of mouse fibroblast cells and outline of fractionation procedure.

from Artisan Metal Industries, Inc., Waltham, Mass. Ficoll (average molecular weight 400,000) and Sephadex G-50 fine were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The Ficoll solution was dialyzed exhaustively against glass-distilled water before use in order to remove salts and other impurities of low molecular weight. ^{3}H- and ^{14}C-labeled glucosamines were obtained from New England Nuclear Corp., Boston, Mass. The [^{14}C]glucose–fructose mixture was prepared from [^{14}C]$BaCO_3$ by the method of Abraham and Hassid (1957).

Cell Cultures. The 3T3 cell line of mouse fibroblasts (Todaro *et al.*, 1964) utilized in the present experiments was obtained from Dr. George Todaro. All studies were carried out with a cloned population of cells. The control 3T3 cells were always passaged prior to confluency in order to ensure against selection of noncontact-inhibited cells. Control 3T3 cells were contact inhibited in that they grew to saturation densities of approximately 7×10^5 cells in a 50-mm plastic pertri dish and remained at this density when maintained for as long as 10 days after confluency had been attained. Cells in the present experiments were derived from the fourth to the eighth passage levels after receiving the cell line.

A 3T3 subline which was less contact inhibited than control cells was produced in the following way. At 4-day intervals the monolayers were dispersed with a trypsin–EDTA solution and one-third of the cells were passed to a new bottle. Confluency was attained in 1–2 days. In this way cells which were less contact inhibited were selected for and saturation densities of approximately 5×10^6 cells/50-mm petri dish were achieved by the 15th passage level. This line will be referred to as spontaneously transformed (ST-3T3) and was utilized at the 29th to 35th passage levels.

SV40 virus-transformed 3T3 (SV-40-3T3) cells were obtained from a transformed colony isolated from semisolid agar (Black, 1966). The transformed cells had a changed morphology, contained the SV40 T antigen as determined by complement fixation and fluorescent antibody procedures, and grew to saturation densities of approximately 10×10^6 cells/50-mm petri dish. SV-40-3T3 cells were utilized at the 4th–11th passage levels after transformation. The medium used for all experiments was composed of Eagle's minimal essential medium with four times the usual concentration of vitamins and amino acids (MEM × 4), 10% fetal calf serum, and penicillin and Creptomycin at concentrations of 250 units and 250 μg/ml, respectively. This media contained 1 g of glucose per liter. The cells were grown in either 32-oz glass bottles which were maintained in a stationary position or on a Bellco roller apparatus at 37°.

Glucosamine-Labeling Experiments and Subcellular Fractionations. The over-all plan of the double-labeling experiments is shown in the diagram in Figure 1. Individual bottles of cells were labeled with either [6-^{3}H]glucosamine (1.5–5 mCi in 3.8–8 mg of glucosamine hydrochloride) or [1-^{14}C]glucosamine (0.5–1.0 mCi in 3.8–8 mg of glucosamine hydrochloride). The label was added in fresh medium 1 day after plating of the cells and was maintained in the medium until the time of harvesting, generally 4–6 days later. Fresh medium was added to the medium containing label 3 or 4 days after plating. The cells were harvested at the time of confluency by pouring off the medium and scraping the cells from the bottles with a rubber policeman into a solution of 0.15 M NaCl in 5 mM Tris (pH 7.4). The cells were then sedimented, washed and fractionated according to the method of Wallach and Kamat (1966). After sedimentation, the cells were washed once in the same solution and once in 0.25 M sucrose, 0.2 mM $MgSO_4$, and 5 mM Tris (pH 7.4). Each centrifugation was at 500*g* for 5 min. The packed cell volumes were then measured and Tris buffer (5 mM, pH 7.4) containing 0.25 M sucrose and 0.2 mM $MgSO_4$ was added to make a cell suspension of 10% (v/v). Aliquots of the cell suspensions were counted for radioactivity and the cells were mixed to form two groups (I and II, Figure 1), each containing approximately equal numbers of counts per minute of ^{3}H and ^{14}C. The two cell mixtures, I and II, were treated separately and the cells were disrupted by equilibration for 20 min at 800 psi in a nitrogen pressure homogenizer followed by a sudden return of the cell suspension to atmospheric pressure.

After expulsion from the nitrogen bomb, the cell homogenates were allowed to stand for 5 min and then were made 0.001 M with respect to Na_2EDTA by the addition of 0.1 M Na_2EDTA (pH 7.4). The cell homogenates were then centrifuged at 600*g* for 10 min to bring down a loose pellet of nuclei. The supernatant fluid was carefully withdrawn from the pellet to avoid drawing up loose material. The pellet was then washed once with 0.25 M sucrose, 0.2 mM $MgSO_4$, and 5 mM Tris (pH 7.4) and the wash was added to the nuclear supernatant. The pellet remaining was designated the nuclear fraction.

The nuclear supernatant fluid was centrifuged at 20,000*g* for 15 min to sediment the mitochondria and the supernatant fluid was again carefully withdrawn. The mitochondria were washed once in 0.25 M sucrose, 0.2 mM $MgSO_4$, and 5 mM

Tris (pH 7.4) and the wash was added to the mitochondrial supernatant fluid. The pellet remaining was the mitochondrial fraction.

The mitochondrial supernatant fluid was centrifuged in the Spinco Model L centrifuge at 104,000*g* for 45 min to bring down a firmly packed microsomal pellet. The microsomal supernatant fluid was withdrawn and fractionated on Sephadex G-50 into two peaks, one excluded and the other included. The microsomal pellet was suspended in a volume of 0.25 M sucrose in 1 mM Tris (pH 8.6) equivalent to that of the packed cell volume with a 0.5-ml plastic disposable syringe equipped with a 25-gauge stainless steel needle. It was then stored in the freezer until needed for isolation of the surface membrane.

Ficoll Barrier Fractionation of the Surface Membrane. The frozen microsomal suspension was thawed and diluted (1 to 5.5) with 0.01 M Tris (pH 8.6), mixed thoroughly, and then centrifuged at 104,000*g* for 30 min. The supernatant fluid was withdrawn and the pellet was resuspended in 1 mM Tris (pH 8.6) to the same volume as the previous step and the centrifugation was repeated. The twice-washed microsomal pellet was suspended in 1.5 ml of 1 mM Tris (pH 8.6) containing 1 mM $MgSO_4$ and homogenized with a Teflon pestle by hand to obtain a uniform suspension. The suspension was then dialyzed twice for 1 hr against 150 ml of 1 mM Tris (pH 8.6)–1 mM $MgSO_4$ at 4°. The dialyzed microsomal fraction was carefully layered on top of 3.0 ml of a Ficoll solution containing 1 mM Tris (pH 8.6) and 1 mM $MgSO_4$ (ρ_4^{25} = 1.0866) and centrifuged in an SW39 rotor in the Spinco Model L-2 centrifuge equipped with a rotor stabilizer. The centrifugation was carried out at 2° for 15 hr at 51,000*g*. After centrifugation a layer of material which contains the surface membrane bands at the interface between the sample solution and the Ficoll. A pellet at the bottom of the tube is the endoplasmic reticulum. The surface membrane fraction was removed by carefully withdrawing the interface layer with a 0.5-ml plastic disposable syringe equipped with a 25-gauge stainless steel needle. The surface membrane and endoplasmic reticulum fractions were both washed with 8 ml of 1 mM Tris (pH 8.6)–1 mM $MgSO_4$ in order to remove Ficoll. Both fractions were resedimented by centrifugation for 5 hr at 2° at 104,000*g*. Each fraction was then adjusted to 2 ml with the same buffer and stored in the freezer.

The degree of cross-contamination of various subcellular fractions is yet to be determined.

Isolation of Amino Sugars and Determination of $^3H/^{14}C$ Ratio. Each fraction was dialyzed against distilled water for 24 hr or more. Aliquots (10 μl) were taken for measuring the $^3H/^{14}C$ ratio of the dialyzed fractions. For the isolation of *N*-acetylneuraminic acid, an aliquot was hydrolyzed in 0.1 N H_2SO_4 at 80° for 1 hr. The hydrolysate was neutralized with 0.4 N $Ba(OH)_2$ to the end point of phenol red. After centrifugation to remove $BaSO_4$, the supernatant fluid was brought to dryness at 45° under a stream of nitrogen. N-Acetylneuraminic acid was isolated by paper electrophoresis for 16–20 hr at 200–300 V in a hanging-strip chamber, the electrophoresis buffer being 0.05 M triethylammonium acetate and 0.1 M acetic acid (pH 4.0). Sialic acid was eluted from the paper, dried and the $^3H/^{14}C$ ratio was measured.

For the isolation of hexosamine, another aliquot was hydrolyzed with 4 N HCl at 100° for 6 hr in a sealed tube. HCl was removed under a stream of nitrogen at 45°. The dried hydrolysate was then taken up in 5 ml of water, which was applied to a 1.1 × 50 cm Dowex 50 (H^+) column for the separation of glucosamine and galactosamine (Gardell, 1953). Elution was effected with 0.3 N HCl and 1-ml fractions were collected. After drying the fractions containing hexosamines under N_2 at 45°, each fraction was counted in 0.25 ml of H_2O and 10 ml of Bray's solution to determine $^3H/^{14}C$ ratios.

Measurements of ^3H and ^{14}C counts were made in either the two-channel Nuclear-Chicago scintillation counter or the three-channel Packard scintillation spectrometer with external standardization; 10 ml of Bray's solution (Bray, 1960) was generally used for counting except in cases of large aliquots of aqueous solutions when 10 ml of Patterson and Greene's scintillation solution containing 33% Triton X-100 was used (Patterson and Greene, 1965).

Chemical Determination of Sugar Composition of Mitochondrial and Microsomal Fractions from 3T3, SV-40-3T3, and ST-3T3 by Isotope Dilution Technique. The contents of the neutral and amino sugars in the subcellular fractions obtained from 3T3, ST-3T3, and SV-40-3T3 cells were determined by isotope dilution according to the method of Shen and Ginsburg (1967).

Labeling of 3T3, ST-3T3, and SV-40-3T3 cells was carried out by growth in the presence of 5 mCi of [^{14}C]glucose-fructose mixture in media containing 5 mM glucose for 4 days. The cells were harvested and separated into their subcellular components as described previously except that no buffers containing sucrose were used in the fractionation. The microsomal fraction was not further separated into surface membrane and endoplasmic reticulum. The cell fraction to be analyzed was first dialyzed against distilled water and then made into a uniform suspension by hand homogenization with a Teflon pestle.

ISOLATION OF [^{14}C]RIBOSE AND DETERMINATION OF SPECIFIC ACTIVITY. Aliquots of microsomal fractions from [^{14}C]glucose–fructose-labeled 3T3, SV-40-3T3, and ST-3T3 were hydrolyzed in 1 N H_2SO_4 for 4 hr at 100° in sealed tubes. No carrier sugar was added. The hydrolysate was neutralized with 0.4 N $Ba(OH)_2$ to the end point of phenol red. After removing $BaSO_4$ by centrifugation, the supernatant solution was deionized by passing through small columns of Dowex 50 (H^+) and Dowex 1 (formate). The water eluate was dried at 45° under a stream of N_2 and the sample was chromatographed on Whatman No. 1 paper twice for the isolation of D-[^{14}C]ribose. The first run was in ethyl acetate–pyridine–water (3.6:1.0:1.15, v/v) for 23 hr (Shen and Ginsburg, 1967); the spots corresponding to ribose were then eluted and rechromatographed in 1-butanol–pyridine–H_2O (6:4:3, v/v) for 23 hr.

Because of the small quantities of [^{14}C]ribose, colorimetric assay was not possible. Specific activity of [^{14}C]ribose was determined indirectly by converting [^{14}C]ribose into [^{14}C]-[^3H]ribitol by reduction with [^3H]$NaBH_4$ of known specific activity. To 1-ml samples containing [^{14}C]ribose was added 100 μl of [^3H]$NaBH_4$ solution (19 mg/ml). After incubation at room temperature for 2 hr, the reaction was stopped by adding 1 ml of Dowex 50 (H^+) resin. The reaction was then deionized by passing it successively through 2-ml columns of Dowex 50 (H^+) and Dowex 1 (acetate). The water elute was dried at 45° under N_2 and was resuspended in methanol three times to remove methyl borate. Ribitol was then isolated by paper chromatography in ethyl acetate–pyridine–

TABLE I: Relative Incorporation of Radioactive Glucosamine into Subcellular Fractions of SV-40-3T3 as Compared with 3T3.

Fraction	I ^3H SV3T3/ ^{14}C 3T3	II ^3H 3T3/ ^{14}C SV3T3	I × II
Homogenate	1.00[a]	1.00[a]	1.00
Nuclei	1.20	0.88	1.06
Mitochondria	0.80	1.25	1.00
Microsomes	0.78	1.29	1.01
Supernatant fluid	1.44	0.64	0.92
soluble glycoproteins	0.66	1.59	1.05
Nucleotide sugars	4.54	0.19	0.86

[a] The $^3H/^{14}C$ ratios of homogenate I and II were found to be 1.08 and 0.93, respectively. The ratios of various subcellular fractions from homogenate I (or II) were normalized to the ratio of homogenate I (or II) which was taken to be unity.

water (3.6:1.0:1.15, v/v) for 23 hr. Paper strips of 4 × 3.2 cm containing radioactive ribitol were cut, eluted, and $^3H/^{14}C$ ratios were measured.

To determine the specific activity of ^3H in $NaBH_4$, reduction was carried out in parallel with [^{14}C]glucose and [^{14}C]-glucosamine of known specific activities. To obtain the specific activities of the latter compounds, [^{14}C]glucose was assayed with glucostat and [^{14}C]glucosamine was determined with the Elson–Morgan test (Rondle and Morgan, 1955). From the specific activities of [^{14}C]glucose and [^{14}C]glucosamine and the $^3H/^{14}C$ ratio of purified [^{14}C-^{3}H]glucitol and 2-acetamido-2-deoxy-D-[^{14}C-^{3}H]glucitol (after N-acetylation of 2-amino-2 deoxy-D-glucitol) (Spiro, 1966), the specific activity of ^3H in $NaBH_4$ was obtained.

DETERMINATION OF *N*-ACEYLNEURAMINIC ACID, *N*-ACETYLGLUCOSAMINE, AND *N*-ACETYLGALACTOSAMINE CONTENT IN MITOCHONDRIAL FRACTIONS OF 3T3, SV-40-3T3, AND ST-3T3. [^{14}C]*N*-Acetylneuraminic acid was isolated from the mitochondrial membrane fraction after acid hydrolysis with 0.05 N H_2SO_4 at 80° for 1 hr, in the presence of 100 μg of *N*-acetylneuraminic acid as carrier. The acid hydrolysate was neutralized with 0.4 N $Ba(OH)_2$ to the end point of phenol red, centrifuged, and the supernatant solution was applied to a 2-ml Dowex 50-X8 (H^+) column followed by a 2-ml Dowex-X8 (formate) column. After washing the columns with water, the Dowex 1-X8 (formate) column was eluted with 15 ml of 0.3 N formic acid. The formic acid eluate was dried under N_2 at 45° and chromatographed on Whatman No. 3MM paper as a 2-in. band in butyl acetate: acetic acid: water (3:2:1,v/v) for 24 hr (Shen and Ginsburg, 1967). The radioactive spots corresponding to standard *N*-acetylneuraminic acid were cut out as 1 × 2 in. strips, eluted with water, and the dried samples were rechromatographed in ethyl acetate–pyridine–acetic acid–water (5:5:1:3, v/v) for 24 hr (Fischer and Nebel, 1955). After scanning for radioactivity, [^{14}C]*N*-acetylneuraminic acid was isolated from the paper. A small aliquot was used for radioactivity assay by scintillation counting; chemical determination of *N*-acetylneuraminic acid was carried out with thiobarbituric acid reagent in duplicate (Warren, 1959). Proper paper blanks were included in the assay.

For the determination of [^{14}C]*N*-acetylglucosamine and *N*-acetylgalactosamine contents in the mitochondrial fractions, aliquots of mitochondrial suspensions were hydrolyzed in sealed tubes with 4 N HCl at 100° for 6 hr in the presence of 300 μg each of carrier sugars (*N*-acetylglucosamine and *N*-acetylgalactosamine). After removing HCl under N_2 at 45°, hexosamines were adsorbed to 2 ml of Dowex 50 (H^+) resin and eluted with 15 ml of 2 N HCl. After repeated drying and resuspension with water to remove HCl, ninhydrin degradation of hexosamine was carried out according to the method of Spiro (1966). After desalting with Dowex 50 (H^+) and Dowex 1 (formate) columns, the water eluate containing [^{14}C]arabinose and lyxose was dried and chromatographed as a 2-in. band on Whatman No. 3MM paper in 1-butanol–pyridine–H_2O (6:4:3, v/v) for 24 hr. Radioactive peaks corresponding to arabinose and lyxose were cut and eluted with water. The dried sample was rechromatographed in ethyl acetate–pyridine–H_2O (3.6:1.0:1.15, v/v) for 28 hr. [^{14}C]-Arabinose and lyxose were isolated from 2 × 3.1 cm strips and measured for radioactivity with scintillation counting and assayed chemically with phloroglucinol reagents (Dische and Borenfreund, 1957).

From the specific activities of isolated *N*-acetylneuraminic acid, arabinose and lyxose and the amount of carrier sugars added, the total counts per minute of *N*-acetylneuraminic acid, *N*-acetylglucosamine, and *N*-acetylgalactosamine present in the original mitochondrial suspension could be calculated. This was converted into micrograms of each sugar by assuming the specific activity of each sugar in the mitochondrial suspension to be equal to that of [^{14}C]ribose isolated from the microsomes.

Protein concentration in the mitochondrial suspension was determined by the Lowry procedure with sodium deoxycholate added at a final concentration of 0.38% (Lowry *et al.*, 1951).

DETERMINATION OF NEUTRAL SUGARS IN MITOCHONDRIAL AND MICROSOME FRACTIONS OF 3T3, SV-40-3T3, AND ST-3T3. To the dialyzed samples were added 300 μg each of D-galactose, D-glucose, D-mannose, and L-fucose. The samples were then made 1 N in H_2SO_4 and hydrolyzed in sealed tubes at 100° for 4 hr. The hydrolysates were neutralized with saturated $Ba(OH)_2$ using phenol red as an indicator. The $BaSO_4$ precipitate was removed by centrifugation and the supernatant solution was deionized by passage through a column composed of half AG 50-X8 (H^+) and half AG 1-X4 (acetate).

The column eluates were concentrated and chromatographed on Whatman No. 3MM paper in ethyl acetate–pyridine–H_2O (3.6:1.0:1.5, v/v) for 20 hr. The areas corresponding to galactose, glucose, mannose, and fucose were eluted from the paper with water, the eluates were concentrated and then rechromatographed in the same solvent. Fucose was rechromatographed for 24 hr, mannose for 48 hr, and galactose and glucose for 68 hr. The areas corresponding to the pure sugars were then eluted with water and after concentration of the eluates, the amount of sugar was determined by colorimetric analysis. Radioactivity of the sample was deter-

TABLE II: Relative Contents of Sialic Acid, Glucosamine, and Galactosamine in Membrane Glycoproteins of SV-40-3T3 and 3T3.

Fraction	I ^{3}H SV3T3/^{14}C 3T3			II ^{3}H 3T3/^{14}C SV3T3		
	N-Acetyl-neuraminic Acid/Gross[a]	Glucos-amine/Gross	Galactos-amine/Gross	*N*-Acetyl-neuraminic Acid/Gross	Glucos-amine/Gross	Galactos-amine/Gross
Nuclei	0.43	1.33	0.57	2.20	0.68	1.38
Mitochondria	0.41	1.54	0.60	2.44	0.65	1.51
Surface membrane	0.45	2.44	0.67	1.86	0.34	1.33
Endoplasmic reticulum	0.42	1.71	0.55	2.09	0.46	1.21
Soluble glycoproteins	0.47	1.08	0.63	2.44	1.00	1.49
Nucleotide sugars	1.32	1.30	1.28	1.10	1.06	0.93

[a] The ratio of *N*-acetylneuraminic acid/gross of a particular fraction was obtained by dividing the ^{3}H/^{14}C ratio of *N*-acetylneuraminic acid isolated from that fraction by the gross ^{3}H/^{14}C ratio of this given fraction. It can be shown that this ratio is equal to per cent of *N*-acetylneuraminic acid in ^{3}H-labeled fraction/per cent of *N*-acetylneuraminic acid in ^{14}C-labeled fraction, thus representing a measurement of *relative* sialic acid contents of glycoproteins in ^{3}H- and ^{14}C-labeled fractions.

mined by counting in a Packard liquid-scintillation counter in Bray's solution.

Fucose was determined by the cysteine–sulfuric acid reaction (Dische and Shettles, 1948). Mannose, glucose, and galactose were determined by the anthrone reaction (Roe, 1955).

Results

Cells of the two cell lines 3T3 and SV-40 transformed 3T3 were labeled with [^{14}C]- and [^{3}H]glucosamine, respectively, and fractionated into nuclei, mitochondria, endoplasmic reticulum, and surface membrane according to the procedure of Wallach. The total incorporations of glucosamine at the end of 5 days were 1 and 2% for 3T3 and SV-40-3T3 cells, respectively. The incorporation of glucosamine into subcellular fractions was measured and ^{3}H/^{14}C ratios were calculated for each fraction. Any preferential incorporation of glucosamine into a particular subcellular fraction of one cell line is reflected in the deviation of the ^{3}H/^{14}C ratio of that fraction from that of the whole homogenate. As shown in Table I, the distribution of ^{3}H and ^{14}C labels in these subcellular fractions of 3T3 and SV-40-3T3 is different, indicating a quantitative difference in the glycoprotein and glycolipid contents of particulate fractions. When the ^{3}H/^{14}C ratios of individual amino sugars isolated from a particular fraction were determined and compared with the gross ratio of that fraction (see footnote *a* for Table II) the results clearly showed that the amino sugar compositions of the fractions from SV-40-3T3 were qualitatively different from those of 3T3. Thus, in the combination of ^{3}H SV-40-3T3 and ^{14}C 3T3, the ^{3}H/^{14}C ratio of *N*-acetylneuraminic acid in each particulate fraction was found to be very much lower than the gross ratio, whereas the ^{3}H/^{14}C ratio of glucosamine was found to be higher than the gross ratio of each fraction (Table II).

These results suggest that the over-all composition of glycoproteins and glycolipids in the membrane fractions of SV-40-3T3 is relatively low in *N*-acetylneuraminic acid and galactosamine and high in glucosamine *as compared with the composition of 3T3 fractions*. This qualitative difference in the glycoprotein composition of 3T3 and SV-40-3T3 membrane fractions is also clearly shown by the data in Table III where the ratio of total glucosamine to total galactosamine of each fraction is compared. This ratio was obtained by integrating the total radioactivity recovered as glucosamine and galactosamine after separation with Dowex 50 (H^+) column chromatography, and the ratio of glucosamine to galactosamine of each isotope was calculated, independent of the ^{3}H/^{14}C ratio. As can be seen from Table III, this ratio was increased by a factor of 2.5 in SV-40-3T3 cells as compared with that in 3T3.

It should be noted that the composition of the nucleotide sugar fractions was identical in 3T3 and SV-40-3T3 even though SV-40-3T3 had more radioactivity in this fraction as compared with 3T3. An interesting and unanticipated finding was the parallel changes in the amino sugar composition in all

TABLE III: Ratio of Glucosamine to Galactosamine in Glycoproteins of Various Subcellular Fractions from 3T3 and SV-40-3T3.[a]

Fraction	SV3T3		3T3	
	I (^{3}H)	II (^{14}C)	I (^{14}C)	II (^{3}H)
Nuclei	8.3	7.4	3.6	3.6
Mitochondria	7.8	6.8	3.0	2.9
Surface membrane	7.8	7.7	2.2	2.0
Endoplasmic reticulum	8.1	8.3	2.6	3.2
Soluble glycoproteins	5.1	4.2	3.0	2.8
Nucleotide sugars	3.1	2.8	3.1	3.2

[a] Glucosamine and galactosamine were separated by Dowex 50 (H^+) column chromatography. The total radioactivities recovered as glucosamine and galactosamine were integrated and the ratio of glucosamine to galactosamine of each isotope was calculated independently of the ^{3}H/^{14}C ratio of either amino sugar.

TABLE IV: Relative Incorporation of Radioactive Glucosamine into Subcellular Fractions of ST-3T3 and 3T3 ^{3}H ST-3T3/^{14}C 3T3.

Fraction	$^{3}H/^{14}C$ Ratio
Homogenate	1.00[a]
Nuclei	0.95
Mitochondria	0.86
Microsome	0.88
Supernatant	1.47
Soluble glycoproteins	1.20
Nucleotide sugars	1.63

[a] The $^{3}H/^{14}C$ ratio of the homogenate was found to be 0.94. The ratios of various subcellular fractions were normalized to the ratio of the homogenate which was taken to be unity.

particulate fractions. This is clearly shown both in the glucosamine/galactosamine ratios and the ratios of individual amino sugar to gross ratio in each fraction. It is possible that whatever the mechanism of the alteration in glycoprotein composition may be, the changes have affected all particulate fractions to similar extents. However, since cross-contamination cannot be ruled out in the fractionation procedure used in the present study, this possibility will be subject to further investigation.

Comparing the results obtained from cell mixture I (^{3}H-labeled SV-40-3T3 plus ^{14}C-labeled 3T3) with those obtained from cell mixture II (^{3}H-labeled 3T3 and ^{14}C-labeled SV-40-3T3), it is clear that the observed difference in the amino sugar composition of the particulate fractions in the two cell lines is not due to any differential metabolism of [6-^{3}H]glucosamine and [1-^{14}C]glucosamine at the radioactively labeled position by mouse fibroblast cells. The reciprocity of $^{3}H/^{14}C$ ratios obtained from mixtures I and II can be seen in Tables I–III.

TABLE V: Relative Contents of Sialic Acid, Glucosamine, and Galactosamine in the Particulate Fractions of ST-3T3 and 3T3 ^{3}H ST-3T3/^{14}C 3T3.

Fraction	*N*-Acetylneuraminic Acid/Gross[a]	Glucosamine/Gross	Galactosamine/Gross
Nuclei	0.96	0.96	0.80
Mitochondria	0.93	0.95	0.71
Surface membrane	1.07	1.52	0.77
Endoplasmic reticulum	0.96	1.11	0.89
Soluble glycoproteins	0.96	0.77	0.76
Nucleotide sugars	0.67	1.06	0.99

[a] See footnote *a* for Table II.

TABLE VI: Ratio of Glucosamine to Galactosamine in Glycoproteins of the Subcellular Fractions from 3T3 and ST-3T3.

Fraction	Glucosamine/Galactosamine[a]	
	^{3}C ST-3T3	^{14}C 3T3
Nuclei	3.5	2.9
Mitochondria	5.1	3.8
Surface Membrane	2.2	1.1
Endoplasmic reticulum	5.6	4.5
Soluble glycoproteins	3.5	3.5
Nucleotide sugars	2.3	2.1

[a] See footnote *a* for Table III.

The observed difference in the amino sugar composition of the particulate fractions of 3T3 and SV-40-3T3 might be correlated with either the presence of the viral genome in the transformed 3T3 cells or the altered growth properties of transformed 3T3 cells in general. The amino sugar composition of the particulate fractions of spontaneously transformed cells which were selected for their relative loss of contact inhibition of cell division, was compared with that of 3T3 by double-labeling [6-^{3}H]glucosamine and [1-^{14}C]glucosamine, respectively. It is obvious from Tables IV–VI that except for its relatively low galactosamine content, the over-all composition of glycoproteins and glycolipids in the membrane fractions of spontaneously transformed 3T3 is similar to that of 3T3. The ratio of glucosamine to galactosamine in all fractions except

TABLE VII: Relative Incorporation of Radioactive Glucosamine into Subcellular Fractions of Growing 3T3 as Compared with Confluent 3T3.

Fraction	^{3}H Growing 3T3/^{14}C Confluent 3T3 A	^{3}H Confluent 3T3/^{14}C Growing 3T3 B	Product A × B
Homogenate	1.00[a]	1.00[a]	1.00
Nuclei	1.82	0.60	1.09
Mitochondria	1.16	0.89	1.03
Microsome	1.34	0.80	1.07
Supernatant	0.81	1.32	1.07
Soluble glycoproteins	0.71	1.84	1.30
Nucleotide sugars	2.95	0.34	1.00

[a] The $^{3}H/^{14}C$ ratios of homogenates A and B were found to be 0.41 and 2.34, respectively. The ratios of various subcellular fractions from homogenate A (or B) were normalized to the ratio of homogenate A (or B) which was taken to be unity.

TABLE VIII: Relative Contents of Sialic Acid, Glucosamine, and Galactosamine in the Particulate Fractions of Growing 3T3 and Confluent 3T3.

Fraction	^{3}H-Growing 3T3/^{14}C Confluent 3T3			^{3}H Confluent 3T3/^{14}C-Growing 3T3		
	N-Acetyl-neuraminic Acid/Gross[a]	Glucos-amine/Gross	Galactos-amine/Gross	*N*-Acetyl-neuraminic Acid/Gross	Glucos-amine/Gross	Galactos-amine/Gross
Nuclei	0.99	1.03	0.78	0.68	0.51	0.72
Mitochondria	0.87	0.85	0.73	0.90	0.59	0.77
Surface membrane	1.12	1.53	0.83	1.05	0.79	1.39
Endoplasmic reticulum	0.91	1.03	0.72	0.73	0.71	0.88
Soluble glycoproteins	0.91	0.65	0.98	0.40	0.90	0.48
Nucleotide sugars	0.43	0.82	0.66	1.33	0.95	1.19

[a] See footnote *a* for Table II.

the soluble fraction is higher in spontaneously transformed 3T3 than in 3T3 (Table VI).

To exclude the possibility that the difference in carbohydrate composition between 3T3 and SV-40-3T3 results from the subtle difference in the growth rate and in the status of contact inhibition of cell division near the end of the labeling period, 3T3 cells were grown both as sparse cultures and at near saturation density and labeled with [^{3}H]- and [^{14}C]glucosamine. The results of this experiment are shown in Tables VIII–X. Though there are minor qualitative differences in the glycoprotein composition between growing 3T3 cells and confluent 3T3 cells, the magnitude of the difference is far too small to account for the observed difference between 3T3 and SV-40-3T3. Thus minor differences in state of confluency could not account for the observed difference in composition between 3T3 and SV-40-3T3.

Determination of the Sugar Contents of Mitochondrial Fractions from 3T3 and Transformed 3T3 cells by the Isotope Dilution Technique. Though the double-labeling experiments have revealed differences in the composition of the membrane-bound carbohydrates from 3T3 and SV-40-3T3, no definitive answer is provided concerning the absolute amounts of each amino sugar in a given fraction of a cell line. To obtain this information, we have employed the isotope dilution technique first applied by Shen and Ginsburg to sugar analysis of HeLa cells.

Cells were grown in the presence of a uniformly labeled [^{14}C]glucose–fructose mixture for four to five generations. After subcellular fractionation, carrier sugars of known quantities were added to an aliquot of dialyzed mitochondrial fraction prior to acid hydrolysis. Radioactive sugar was isolated by paper chromatography, purified and its specific activity was determined. Assuming that the specific activity of each neutral and amino sugar is equal to that of ribose, we can calculate the amount of each sugar originally present in a given fraction of each cell line. For the determination of the specific activity of ribose, [^{14}C]ribose was isolated from acid hydrolysates of microsomal fractions without carrier sugar, and was reduced with [^{3}H]$NaBH_4$ of known specific activity to [^{14}C-^{3}H]ribitol. The specific activities of [^{14}C]ribose were calculated from the ^{3}H/^{14}C ratios of ribitol and were found to be 4739 cpm/μg for 3T3, 5108 cpm/μg for SV-40-3T3, and 4545 cpm/μg for ST-3T3.

Based on these values, the compositions of mitochondrial and microsomal fractions of 3T3, SV-40-3T3, and ST-3T3 were calculated as shown in Table X.

It can be seen that in the case of the mitochondria all of the sugars were present in smaller amounts in both the viral and spontaneously transformed cells than in normal 3T3. With the exception of galactose, the SV-40-3T3 cells contained markedly decreased amounts of both amino and neutral sugars.

In the microsome fractions also, there was less neutral sugar in the SV-40-3T3 and ST-3T3 cells than in normal 3T3. The sole exception was galactose which was present in greater amounts in the viral-transformed 3T3 cells than in the other two cell lines. Qualitatively the composition of the neutral sugars in the microsomal fractions of 3T3 were quite similar except for glucose which is present in much smaller amounts in the ST-3T3 cells. This is true also in the SV-40-3T3 cells and is probably due to the absence of glycogen in these transformed cell lines.

TABLE IX: Ratio of Glucosamine to Galactosamine in Glycoproteins of Particulate Fractions from Growing 3T3 and Confluent 3T3.

Fraction	Glucosamine/Galactosamine[a]			
	Growing 3T3		Confluent 3T3	
	^{3}H	^{14}C	^{3}H	^{14}C
Nuclei	5.7	5.6	4.0	4.3
Mitochondria	3.8	4.4	3.4	3.3
Surface membrane	3.9			2.1
Endoplasmic reticulum	5.0			3.5
Soluble glycoproteins	7.6			11.4
Nucleotide sugars	3.1	2.4	2.0	2.5

TABLE X: Sugar Contents of "Mitochondrial" and "Microsomal" Fractions of 3T3, SV-40-3T3, and ST-3T3 as Measured by Isotope Dilution Technique (μg of sugar/mg of protein).

Fraction	*N*-Acetyl-neuraminic Acid	*N*-Acetyl-glucosamine	*N*-Acetyl-galactos-amine	Fuc	Man	Gal	Glu
3T3 mitochondria	10.2	11.7	4.0	1.5	9.8	18.3	3.1
SV 40 3T3 mitochondria	2.4	4.8	0.8	0.9	5.3	14.8	1.3
ST 3T3 mitochondria	7.0	6.9	1.6	1.3	8.5	15.3	1.6
3T3 microsomes				0.8	5.2	11.2	67
SV 40 3T3 microsomes				0.5	3.8	18.5	0.6
ST 3T3 microsomes				0.3	2.0	4.6	3.1

Discussion

There are several lines of evidence which indicate the biological significance of carbohydrates in cell membranes. It has been postulated that the H-2 or histocompatibility antigens (Shimada and Nathenson, 1967; Nathenson, 1968) and the blood-group substances (Watkins, 1967) are composed of glycoproteins and glycolipids. Removal of sialic acid by treatment with neuraminidase destroys cell surface receptors which, are essential for the adsorption of certain viruses (Gottschalk, 1959). Recently, evidence has been presented which suggests that mammalian cells grown *in vitro* in the presence of different sugars have changed morphologies and assume different growth properties (Cox and Gesner, 1965, 1967). Moreover, tight cell-to-cell contact and adhesion may also be dependent upon sugars or glycoproteins of the cell surface, as indicated by the effect of removal of sialic acid from mammalian cells on cell aggregation (Kemp, 1968) and by the participation of specific glycoproteins in the mating of yeast (Crandall and Brock, 1968). While these studies encompass a broad range of biological phenomena, they suggest that the glycoproteins of the cell surface may be important mediators of growth restraint.

Previous studies have revealed differences in the sugar composition of normal and virus transformed cells. Thus, less sialic acid is present in cells transformed by at least two oncogenic DNA viruses (Ohta *et al.*, 1968). Hakamori and Murakami (1968) have shown that following transformation of hamster fibroblasts by polyoma virus, there is a decrease in the major glycolipid hematoside, *N*-acetylneuraminyl lactosyl ceramide, with a concomitant increase in lactosyl ceramide. An antigenic reactivity characteristic of the Forssman antigen, a complex glycolipid, appears in virus-transformed cells which lack this antigen prior to transformation (Fogel and Sachs, 1962; Robertson and Black, 1969; O'Neil, 1968). These changes in antigenicity and sugar composition of the cell surface following transformation by oncogenic viruses are reminiscent of lysogenic conversion of *Salmonella anatum* by temperate bacteriophages ϵ^{15} and ϵ^{34} (Robbins and Uchida, 1962).

The studies presented herein have revealed pronounced differences between normal and SV-40 virus-transformed mouse cells. By analyzing differences in carbohydrate contents of the particulate cell fractions, marked quantitative differences in sugar content were disclosed in that most neutral and amino sugars, in particular sialic acid and *N*-acetylgalactosamine, were found to be lower in transformed cells. When individual amino sugars from each fraction were analyzed and compared with the total amino sugar content of the fraction, qualitative differences in the amino sugar composition of the fractions were present as well. Moreover, the changes found were reflected in all the particulate fractions of the transformed cell. Thus, there appears to be a defect in the transformed cell which is manifested by lower neutral and amino sugars in all the membranes of the cell.

The question arises whether the changes found are specific for the virus causing the transformation. Since similar, though not as extensive, changes were present in spontaneously transformed 3T3 cells, it is more likely that the changes observed accompany the assumption of a more autonomous state of the cell. One naturally wonders whether the observed differences in carbohydrate content of the plasma membrane may be associated with the loss of contact inhibition. This is not known at present. In order to answer some of these questions, similar studies will have to be carried out with other cell lines and other oncogenic viruses as well as cells acutely infected with these oncogenic viruses. Of particular interest in this respect are those "revertants" of virus-transformed 3T3 cells which have regained contact inhibition of cell division (Pollack *et al.*, 1968).

The changes in sugar composition of transformed cell membranes may be explained by a modification of the mechanisms of sugar utilization by the transformed cell line at any number of stages from uptake of glucose to the insertion of neutral or amino sugars on membrane receptors. It is possible that the amount of glucose available for synthesis of glycoproteins and glycolipids is limited in transformed cells. Such a limitation could be brought about by a diminished permeability or by an excessive rate of aerobic glycolysis. In addition, the mechanism for conversion of glucose into nucleotide sugar derivatives could be defective in transformed cells. The fact that the nucleotide amino sugar fractions in both normal, spontaneous and virus-transformed cells are the same argues against these hypotheses. This defect could be a manifestation of a deficiency in the transferase enzymes *per se* or could be the result of changes in the structure of the membrane proteins that are normally glycosylated. Transferase enzymes which carry out this function have not been studied in the present system as

yet. However, it is noteworthy that the activity of a collagen–glucosyl transferase enzyme in 3T3 fibroblasts transformed by either polyoma or SV-40 viruses has been found to be distinctly lower than in nontransformed cells (Bosmann and Eylar, 1968). The malfunctioning of the transferase enzyme systems may explain the lower sialic acid contents of the plasma membranes of transformed cells reported here and elsewhere (Ohta *et al.*, 1968) as well as the appearance of the Lewis or H blood group factors in the malignant neoplasms of patients with blood group A (Hakamori *et al.* 1967). In both of these instances it could be postulated that similar core structures are present in the glycolipids and glycoproteins of the transformed cell but that peripheral sugars such as sialic acid, galactosamine, or fucose are not added to the basic core structure. Although our findings of decreased quantities of neutral and amino sugars in transformed cell membranes are consistent with this hypothesis, other data presented in the accompanying paper indicate that the situation may be more complex.

Acknowledgments

The authors are indebted to Mrs. Vreni Mueller, Miss Margaret Tanner, and Miss Cynthia Dixon for excellent technical assistance.

References

Abraham, S., and Hassid, W. Z. (1957), *Methods Enzymol. 4*, 489.

Black, P. H. (1966), *Virology 28*, 760.

Bosmann, H. B., and Eylar, E. H. (1968), *Nature 218*, 582.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Cox, R. P., and Gesner, B. M. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1571.

Cox, R. P., and Gesner, B. M. (1967), *Cancer Res. 27*, 974.

Crandall, M. A., and Brock, T. D. (1968), *Science 161*, 475.

Defendi, V., and Gasic, G. (1963), *J. Cellular Comp. Physiol. 62*, 23.

Dische, Z., and Borenfreund, E. (1957), *Biochim. Biophys. Acta 23*, 639.

Dische, Z., and Shettles, L. B. (1948), *J. Biol. Chem. 175*, 595.

Fischer, F. G., and Nebel, H. J. (1955), *Z. Physiol. Chem. 302*, 10.

Fogel, M., and Sachs, L. (1962), *J. Natl. Cancer Inst. 29*, 239.

Forrester, J. A., Ambrose, E. J., and Macpherson, I. A. (1962), *Nature 196*, 1068.

Forrester, J. A., Ambrose, E. J., and Stoker, M. (1964), *Nature 221*, 945.

Gardell, S. (1953), *Acta Chem. Scand. 7*, 207.

Gottschalk, A. (1959), *in* The Viruses, Vol. III, Burnet, F. M., and Stanley, W. M., Ed., New York, N. Y., Academic, p 51.

Habel, K. (1961), *Proc. Soc. Exptl. Biol. Med. 106*, 722.

Hakomori, S., Koscielak, J., Bloch, K. J., and Jeanloz, R. W. (1967), *J. Immunol. 98*, 31.

Hakomori, S., and Murakami, W. T. (1968), *Proc. Natl. Acad. Sci. U. S. 59*, 254.

Kemp, R. B. (1968), Nature *218*, 1255.

Kornfeld, S., and Ginsburg, V. (1966), *Exptl. Cell Res. 41*, 592.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Nathenson, S. G. (1968), *in* Biological Properties of the Mammalian Surface Membrane, Manson, L. A., Ed., Philadelphia, Pa., The Wistar Institute, p 116.

Ohta, N., Pardee, A.B., McAuslan, B. R., and Burger, M. M., (1968), *Biochim. Biophys. Acta 158*, 98.

O'Neill, C. H. (1968), *J. Cell. Sci. 3*, 405.

Patterson, M. S., and Greene, R. C. (1965), *Anal. Chem. 37*, 854.

Pollack, R. E., Green, H., and Todaro, G. (1968), *Proc. Natl. Acad. Sci. U. S. 60*, 126.

Robbins, P. W., and Uchida, T. (1962), *Fed. Proc. 21*, 702.

Robertson, H. T., and Black, P. H. (1969), *Proc. Soc. Exp. Biol. Med.* (in press).

Roe, J. H. (1955), *J. Biol. Chem. 212*, 335.

Rondle, C. J. M., and Morgan, W. T. J. (1955), *Biochem. J. 61*, 586.

Shen, L., and Ginsburg, V. (1967), *Arch. Biochem. Biophys. 122*, 474.

Shimada, A., and Nathenson, S. G. (1967), *Biochem. Biophys. Res. Commun. 29*, 828.

Sjögren, H. O., Hellstrom, I., and Klein, G. (1961), *Cancer Res. 21*, 329.

Spiro, R. G. (1966), *Methods Enzymol. 8*, 3.

Todaro, G. J., Green, H., and Goldberg, B. D. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 66.

Wallach, D. F. H., and Kamat, V. B. (1966), *Methods Enzymol. 8*, 164.

Warren, L. (1959), *J. Biol. Chem. 234*, 1971.

Watkins, W. M. (1967), *in* The Specificity of Cell Surfaces, Davis, B. D., and Warren, L., Ed., Englewood Cliffs, N. J., Prentice Hall, p 257.