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Glycosyltransferase and Sialic Acid Levels of Normal and Transformed Cells†

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ABSTRACT: Ability to catalyze transfer of sialic acid, fucose, and galactose to exogeneous acceptors was measured in extracts from a wide variety of normal and transformed cells. Sialic acid transferring ability was reduced when normal mouse cells are transformed by Simian virus 40 (SV40), and hamster cell lines are transformed by polyoma virus (Py) or hamster sarcoma virus. Flat revertants of SV40-transformed cells have increased while spontaneous transformed cells have decreased sialic acid transferring ability. Py virus transformed mouse cells were the only transformed cell line which did not have decreased sialic acid transferring ability. Galactose and fucose transferring ability was reduced when some cell lines were transformed, but was unaltered in several others. Sialic acid levels of all cell lines were found to be directly related to sialic acid transferring ability measured in cell extracts.

The cell surface has long been postulated to function in the regulation of cell growth. A correlate of this postulate is that cell surface alterations will lead to loss of the regulatory control of cell division. Biochemical studies of cell surface glycoproteins and glycolipids have revealed alterations associated with transformation.

Experiments measuring relative compositions of glycoproteins from 3T3 and SV40-transformed 3T3 cells indicate reduced carbohydrate levels in the transformed cells (Wu *et al.*, 1969). Chemical measurements show that cells transformed by RNA and DNA viruses have reduced levels of sialic acid (Ohta *et al.*, 1968; Grimes, 1970; Perdue *et al.*, 1972). Evidence has been published that glycoprotein structures are altered by transformation (Meezan *et al.*, 1969; Buck *et al.*, 1971; Onodera and Sheinin, 1970). Biochemical studies on glycolipids have shown that in hamster cells, hematoside, the Forssman antigen, and ceramide trihexoside are reduced in transformed cells as compared with normal cells (Hakamori and Murakami, 1968; Hakomori, 1970; Robbins and Macpherson, 1971). In cultured cells derived from mouse embryos, the complex gangliosides of normal cells are lacking in transformed cells (Mora *et al.*, 1969).

Alterations in complex carbohydrate compounds are accompanied by changes in the glycosyltransferases. Transformed hamster cells have reduced levels of the α-galactosyltransferase which catalyzes synthesis of ceramide trihexoside (Kijunoto and Hakomori, 1971). Transformed mouse cells have reduced levels of a *N*-acetyl-D-galactosaminyltransferase which catalyzes synthesis of Tay Sachs ganglioside (Cumar *et al.*, 1970). Altered transferase levels accompany cell density dependent changes in glycolipid patterns (Hakomori, 1970; Robbins and Macpherson, 1971; Kijunoto and Hakomori, 1971). Bosmann *et al.* (1968a,b) reported increased glycoprotein glycosyltransferase activities in transformed cells. The ability to catalyze sialic acid transfer to hematoside, monosialyl ganglioside, and desialized glycoprotein acceptors has been reported to be decreased in transformed cells (Grimes, 1970; Den *et al.*, 1971; Grimes and Robbins, 1972). Warren *et al.* (1972) have observed that ability to catalyze sialic acid transfer to fucose containing glycopeptides is elevated in transformed cells. To determine the generality of some of these observations we have studied sialic acid, galactose, and fucose transferases and sialic acid levels in a large variety of normal and transformed cells.

Materials and Methods

Cell Lines. Cell lines used in experiments described have the following origin: 3T3B, SV3T3B, were a gift of Dr. Paul Black, Massachusetts General Hospital. 3T3B is a cloned line of the original Swiss 3T3 cells (3T3G) from Howard Green (see below). SV3T3B is a cell line derived from the cloned line 3T3B after transformation by SV40. ST3T31B is a spontaneous transformant of 3T3B which is less sensitive to contact inhibition. ST3T32B is a second spontaneous transformant cell line and was a gift of Dr. Loyd Culp at Massachusetts General

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Hospital. FLSV1B and FLSV2B are flat revertants isolated from survivors of SV3T3B cells treated with 5-fluoro-2′-deoxyuridine (Culp *et al.*, 1971). A31 is a cloned line of Balb/c 3T3 cells. SVT2 is SV40-transformed A31 cells. A31 and SVT2 were a gift of Dr. George Todaro of N. I. H. Nil b is a clone of Nil cells isolated by Dr. Hisako Sakiyama, M. I. T. (Sakiyama *et al.*, 1972). HSV Nil b is hamster sarcoma virus (HSV) transformed Nil b cells. Transformation was achieved by infecting cells with HSV in the presence of DEAE-dextran (Duc Nguyen, 1968). Transformed cells forming colonies in soft agar were isolated as described (Macpherson and Montagnier, 1970). Py Nil b is a Py virus transformed Nil b cell line and was a gift of Dr. Hisako Sakiyama, M. I. T. BHK cells and Py virus transformed BHK cells were gifts of Dr. Walter Eckhart, Salk Institute. 3T3G is the Swiss line of 3T3 cells and a gift of Dr. Howard Green, M. I. T. Py 3T3G and SV3T3G are Py virus and SV40-transformed 3T3G cells and were gifts of Dr. Green. FLSVG is a flat revertant isolated from 3T3G cells by Dr. Robert Pollock, and was a gift of Dr. Green (Pollock *et al.*, 1968).

All cells were grown in media composed of Eagle's minimal essential media with four times the usual concentration of vitamins and amino acids (4 × MEM), 10% fetal bovine serum, and penicillin and streptomycin at concentrations of 76 units and 50 μg/ml, respectively. The media contained 1 g of dextrose/l. Cells were grown at 37° in Bellco roller bottles with 1400 cm^2 of cell growing area.

Cell lines were routinely tested for *Mycoplasma* by spreading cell suspensions on 2.5% PPLO agar (Difco Laboratories, Detroit, Mich.) containing 20% horse serum and 1% yeast extract (Grand Island Biological Co., Berkeley, Calif.). After incubations for up to 2 weeks, plates were examined in a phase-contrast microscope for PPLO colonies. As indicated previously, sensitive test showed that SV3T3B, ST3T3B, and FLSV1, and 2B were contaminated with *Mycoplasma*. No *Mycoplasma* contamination in any of the other cell lines used in these reported experiments could be detected. *Mycoplasma* is routinely screened once a month during the course of experiments. Cell lines were used for only five to six passages and then fresh low passage cells were started from a nitrogen freezer to minimize either contamination or changes in cell population occurring during cell growth.

Preparation of Substrate. CMP-[^{3}H]sialic acid (25 Ci/mol), CMP-[^{14}C]sialic acid (5 Ci/mol), GDP-L-[^{3}H]fucose (4 Ci/mmol), and UDP-[^{14}C]galactose (64 Ci/mol) were prepared as described previously (Grimes, 1970; Grimes and Robbins, 1972).

Preparation of Acceptors. Desialized bovine submaxillary mucin and fetuin were prepared as described (Grimes, 1970). Galactose and fucose acceptors were prepared by treating 200 mg of desialized fetuin (Sigma Chemical Co., St. Louis) with 40 mg of crude Almond Emulsin (Sigma) for 24 hr, at 37° in the presence of toluene and 0.02 M KPO_4^{2-} (pH 7.0). More than 70% of the galactose of desialized fetuin and less than 10% of the *N*-acetyl-D-glucosamine was removed. The crude enzyme also contains proteases which may cause clevage of some of the peptide portion of fetuin. However, this cleavage is slight since the β-galactosidase-treated fetuin elutes similarly to native fetuin when chromatographed through Sephadex G-100. After enzyme digestion, the mixture was washed through a 2.5 × 25 cm column of DEAE-cellulose (DE-52, equilibrated with 0.02 M KPO_4^{2-}). The column was eluted with buffer and then a linear gradient of 0–1 M KCl in buffer. β-Galactosidase and free galactose were found in the excluded volume from the column while the carbohydrate and protein of fetuin were eluted at a salt concentration of 0.3 M. This preparation served as acceptor in both fucosyl and galactosyl transferase reactions.

Enzyme Preparation and Measurements of Glycosyltransferase Activities. Enzymes were prepared by procedures described previously (Grimes, 1970). Cell monolayers were washed with 50 ml of solution A (0.8% NaCl–0.05% KCl–0.001 M KPO_4, pH 7.4) containing 10^{-3} M EDTA. Cells were removed with the aid of a rubber policeman in 25 ml of solution A. The cell pellet was suspended in a volume of 0.01 M KPO_4 (pH 6.5) to give 0.6 ml of packed cells/10 ml of buffer. After standing for 10 min, cells were disrupted by equilibration for 20 min at 800 psi in a nitrogen pressure homogenizer followed by rapid return of the cell suspension to atmospheric pressure. After 10 min in ice, unbroken nuclei were removed by centrifugation for 10 min at 600*g*. The supernatant solution was removed and subjected to centrifugation at 39,000 rpm in a Spinco SW40 rotor for 1 hr. The resulting pellet was suspended by homogenization in 2 ml of 0.05 M sodium cacodylate buffer containing 1 mM $MgCl_2$ and 0.1% Triton X-100 (pH 6.5) (buffer I). Conditions for transferase assays were optimal for pH, detergent and detergent concentration, and incubation temperature. Incorporation was usually measured by precipitating glycoprotein products with 0.5 N HCl containing 1% phosphotungstic acid. Incorporations were also measured by counting immobile radioactivity when incubated reactions were electrophoresised in 0.02 M potassium borate (pH 8.0) or chromatographed against ethanol–1 M ammonium acetate (pH 7.6) (70:35). Results from the three assay procedures were the same. Galactosyltransferase reactions were run in the presence of 20 mM Mn^{2+}, which is an absolute requirement and optimal for galactosyltransferases.

Sialic acid levels were measured on cells prepared by two methods. In the first method, cells grown in Bellco roller bottles were washed with 50 ml of solution A containing 100 mg/l. of $CaCl_2$ and 133 mg/l. of $MgCl_2$ $(H_2O)_2$ (PBS). The cells were removed in 25 ml of PBS with the aid of a rubber policeman and pelleted by centrifugation at 1000 rpm for 5 min. The cell pellet was washed in 25 ml of PBS and again centrifuged. In the second method, cells were rinsed with 50 ml of PBS and then 50 ml of solution A containing 10^{-3} M EDTA was added. The cells were placed back on the roller apparatus for 10 min at which time most of the cells were either floating or were easily detached by rapidly rotating the flask. Cells were collected by centrifugation as above and were washed once with 25 ml of solution A containing 10^{-3} M EDTA and recentrifuged. The latter method was adapted following the observation (W. J. Grimes, unpublished observations) that the rubber policeman technique removes around 12% while the EDTA procedure removes less than 5% of the trichloroacetic acid precipitable radioactivity in cells grown in the presence of [^{14}C]glucosamine. When the second procedure was followed, EDTA washings of cells contained less than 5% of the sialic acid of cells. Both procedures yielded essentially identical values for cellular sialic acid levels.

Cell pellets prepared by either method contained about 2–4 mg of protein. Pellets were suspended in 1 ml of H_2O and the pH was adjusted to 1.0 by the addition of 5-μl aliquots of 5 N H_2SO_4. Cells were hydrolyzed at 85° for 1 hr. This method produced maximal yields of sialic acid. Sialic acid was measured on 0.1-ml aliquots by the thiobarbituric acid method (Warren, 1959). The contribution of 2-deoxyribose to the adsorption at 549 mμ was eliminated by measuring absorption at 532 mμ. In about one-third of the measurements, sialic acid was purified by passing an aliquot of the hydrolysate through a column

of Dowex 1 (formate) (Spiro, 1966). Sialic acid was eluted with 0.3 N formic acid as described. Results from experiments using sialic acid purified by chromatography on Dowex 1 were essentially the same as results obtained using the crude hydrolysate.

Proteins were measured by the method of Lowry *et al.* (1951).

Saturation Density. Saturation densities were measured by plating cells on tissue culture dishes at densities of 3–5 $\times$ 10^5 cells/20 cm^2 growing area. Medium was changed every other day until cells became dense, and every day after confluency was achieved. The saturation density is defined as the density at which the increase in cell number is no longer apparent, and the total number of cells is stable for 3–4 days. Most of the transformed cells never reached a complete plateau, but after the cells became several layers thick, cells detach and die at about the same rate as new cells divide.

The rate of cell growth was approximately the same for most of the cells studied (about 16–18 hr). Hamster cells tended to divide with a slightly faster cell generation time (about 14 hr).

Results

Incubation of cellular particulate extract with either GDP–[^{3}H]fucose, UDP–[^{14}C]galactose, or CMP–[^{3}H]sialic acid and the appropriate acceptor resulted in transfer of radioactivity to a product that was precipitable with 1% phosphotungstic acid in 0.5 N HCl. Enzyme reaction conditions were optimized for the method of enzyme preparation, pH, detergent concentration, and reaction temperature. Studies on the properties of sialic acid transferases were performed on extracts from all of the mouse 3T3 cell lines and Nil hamster cell lines, and transformed cell lines derived from them. In these systems, the sialic acid transferase had similar pH optima, heat sensitivity, acceptor specificity, and detergent requirements. No differences in enzyme properties could be detected between any of the cell lines. Galactosyltransferase activity showed an absolute requirement for $MnCl_2$ with maximum enzyme activity at a concentration of 20 mM. No differences in the Mn^{2+} requirement was detected in extracts from normal or transformed cells. No soluble galactosyl-, fucosyl-, or sialyltransferases could be detected. Radioactivity in products ranged from 2000 to 40,000 cpm. Incorporation into acid precipitable products was absolutely dependent on addition of acceptor. Omission of acceptor reduced incorporations to a level that was barely above boiled controls, or about 100–200 cpm.

Ability to catalyze sialic acid and galactose transfer to glycoprotein acceptors was a linear function of incubation time and enzyme concentration from 0.04 to 0.4 mg of enzyme protein per reaction in extracts from all Nil hamster and mouse cell lines. Fucose incorporation into acid precipitable products was a linear function of the incubation time, but did not always give a linear correspondence with enzyme concentration. We have reported detecting a fucosidase activity in extracts of the normal and transformed cells, and the enzyme activity measured is likely a function of both transferase and glycosidase. No substrate breakdown or product breakdown could be detected in galactosyl- and sialyltransferase reactions. We have previously observed that sialic acid transferring ability using desialized bovine submaxillary mucin and fetuin as acceptors was reduced when 3T3 B cells were transformed by SV40 (SV3T3B) (Grimes, 1970). We have also reported that transformation of Balb/c cells (A_{31}) by SV40 (SVT2) leads to reduction of sialic acid transferring ability (Grimes and Robbins, 1972). In both of those studies sialic acid levels per milligram of protein decreased concomitantly with enzyme levels on SV40 transformation. Results shown in Table I summarize studies of transferase levels, sialic acid levels, and saturation densities of several established lines of mouse and hamster cells, and transformed cell lines derived from them. The specific activities reported for glycosyltransferase measurements are the average of from three to ten separate enzyme preparations. Measurements of activities in a single enzyme preparation were generally in duplicate. Errors were almost always less than 10% between duplicates and between enzyme preparations. Enzyme specific activities were the same whether preparations were from cell lines grown at low or high cell density. Growing cells yielded enzyme preparations with the same specific activities as nondividing confluent cells. This result contrasts with glycolipid transferase studies where transferase specific activities were dependent on cell density (Kijunoto and Hakomori, 1971). Sialic acid levels of various cell lines were routinely measured in duplicate on from three to eight separate cell preparations. Preparations from hamster cell lines, Nil b, HSV Nil b, and Py Nil b showed the greatest variance between replicate experiments. Sialic acid levels were very reproducible between separate preparations of the same cell line. Preparations from cells grown at various cell densities and from growing and confluent cells did not affect the sialic acid level obtained.

Comparisons between sialic acid transferase activities of normal and transformed cells are shown in the data of Figure 1. The cell lines have been divided into cells with low saturation density or normal cells, cells with intermediate saturation density, including spontaneous transformants and HSV-transformed cells, and transformed cells with high saturation densities. Comparing sialic acid transferring ability (Figure 1) reveals that with one exception, a direct correlation exists between sialic acid transferase levels and cell saturation densities. In three normal mouse lines, two from Swiss mice (3T3B and 3T3G) and one from Balb/c mice (A_{31}), transformation by SV40 leads to reduction of sialic acid transferring ability. Spontaneous transformants of the normal cell line also show reduced sialic acid transferring ability. The flat revertant of SV3T3G has a transferase level intermediate between 3T3G and SV3T3G. Other flat revertants (FISV1B) yielded similar results. Transformation of Nil b by HSV and Py virus caused sialic acid transferase activity to decrease. Py BHK cells had reduced sialic transferase levels compared to the parent cell line, but BHK cells yielded preparations with low levels of activity making comparisons difficult. By the criterion of sialic acid transferase and by saturation density, BHK cells would be considered to be transformed. Py 3T3G was a notable exception to the finding that sialic acid transferase levels are reduced when cells lack density-dependent regulated growth. The transferase activities are shown from experiments in which desialized bovine submaxillary mucin was used as acceptor. Similar results were obtained in all cell lines when desialized fetuin was used as acceptor. We have published evidence that the same transferase catalyzes sialic acid transfer to both glycoprotein acceptors (Grimes and Robbins, 1972).

Measurements of galatosyltransferase activities in cell extracts did not show any relationship with transformation (Table I). In 3T3G and Py virus and SV40-transformed 3T3G cells, a reduction in galactosyltransferase was observed. However, the flat revertant FLSVG had galactose transferase levels which were similar to SV3T3G cells. SV40-transformed Balb/c cells (SVT2) had only slightly reduced enzyme levels compared to the parent cell line (A_{31}). Nil b, and the HSV- and

TABLE I: Sialic Acid, Saturation Density, and Glycosyltransference Activities of Normal and Transformed Cells.[a]

Cell Line	B^-: Sialyl[b]	%	F^{2-}: Gal[b]	%	F^{2-}: Fucosyl[b]	%	Sialic Acid	%	Sat. Density (Cells/20 cm² at Confluency)
3T3B	27,600	(100)			86,000	(100)	5.0 ± 0.6	(100)	1.5×10^6
ST3T31B	19,600	71					3.4 ± 0.2	68	6×10^6
ST3T32B							3.3 ± 0.0	66	6×10^6
SV3T3B	15,120	55			50,800	59	3.0 ± 0.6	60	20×10^6
FLSV1B							4.4 ± 0.6	88	2.5×10^6
FLSV2B							4.8 ± 0.3	96	1.6×10^6
A31	30,760	(100)	20,470	(100)	28,720	(100)	6.10 ± 0.2	(100)	1×10^6
SVT2	11,680	38	16,133	79	33,166	117	2.16 ± 0.1	35	20×10^6
Nilb	32,890	(100)	13,666	(100)	127,380	(100)	4.9 ± 1.0	(100)	1.5×10^6
HSVNilb	19,086	58	13,800	101	113,444	89	3.9 ± 1.0	79	7×10^6
PyNilb	13,766	42	14,100	103	61,830	49	2.4 ± 1.0	49	20×10^6
PHK	9,012	(100)					2.5 ± 0.3	(100)	8×10^6
PYBHK	4,966	55					1.8 ± 0.1	72	20×10^6
3T3G	35,100	(100)	45,400	(100)			4.5 ± 0.5	(100)	1.0×10^6
SV3T3G	9,420	27	28,500	63			1.9 ± 0.1	42	20×10^6
Py3T36	30,900	88	32,800	72			4.5 ± 0.5	101	20×10^6
FLSVG	22,800	65	25,600	56			4.1 ± 0.3	92	2.0×10^6

[a] Enzyme preparations from the indicated cells were assayed for ability to catalyze transfer of sialic acid, galactose, and fucose to desialized bovine submaximally mucin (B^-) or desialized β-galactosidase-treated fetuin (F^{2-}). Enzyme assay procedures and methods of preparation are described under Materials and Methods. Descriptions of the various kinds of cells are found under Materials and Methods. For fucosyltransferase, reactions contained 50 μl of enzyme extract in buffer I, 50 μl of a solution of desialized β-galactosidase-treated fetuin (10 mg/ml) in 0.1% Triton X-100, and 5 μl of GDP-[^{3}H]fucose (100,000 cpm at a specific activity of 4 Ci/mmol). For galactosyltransferase, reactions contained 50 μl of enzyme extract in buffer I, 50 μl of desialized β-galactosidase-treated fetuin in 0.1% Triton X-100, 5 μl containing 25,000 cpm of UDP-[^{14}C]galactose at a specific activity of 64 Ci/mol, and 10 μl containing 1.0 M Tris-acetate buffer (pH 7.3) and 0.2 M $MnCl_2$. For sialic acid, reactions contained 50 μl of enzyme extract in buffer I, 50 μl containing 10 mg/ml of desialized bovine submaximally mucin, and 5 μl of CMP-[^{3}H]sialic acid (100,000 cpm at a specific activity at 25 Ci/mol). All incubations were for 1 hr. Galactosyl- and fucosyltransferase reactions were incubated at 37° and sialyltransferase reactions at 26°. Comparisons between cell lines for fucose transferase activity was always made using similar amounts of protein per reaction for the normal and transformed cells. Galactose and sialic acid transferase measurements were made at enzyme concentrations where incorporation was shown to be linear. The values shown are an average of from three to ten measurements, each on fresh enzyme preparations. Incorporations in the absence of acceptor have been subtracted. The normal cell lines are arbitrarily given values of 100% for comparing relative enzyme levels. Procedures for sialic acid levels and cell saturation densities are described under Methods. Sialic acid levels were measured on from three to eight separate cell preparations. The value for sialic acid levels is shown as ± the difference between the average value and that measurement which showed the greatest deviation from the mean. [b] cpm/mg of enzyme protein. [c] μg/mg of protein.

Py virus transformed Nil b cells were indistinquishable with respect to galactosyltransferase levels.

Fewer cells were assayed for the specific activity of fucosyltransferases (Table I). However, the results obtained were similar to galactosyltransferase level studies. Some transformed cell lines show reduced activities compared to parent lines (Py Nil b, SV3T3B) but others do not (SVT2, HSV Nil b). Results of fucosyltransferase experiments are difficult to interpret because of an active fucosidase in enzyme preparations. It is not known whether activities measured for fucosyltransferases are proportional to enzyme levels, fucosidase levels, or both.

Comparisons of sialic acid levels between various cell lines again reveals a striking correlation between sialic acid levels and transformation (Figure 2). The three normal mouse cell lines all showed decreased sialic acid levels upon transformation by SV40 (SVT2, SV3T3B, SV3T3G) or spontaneous transformation (ST3T31B, ST3T32B). Flat revertants (FLSV1B, FLSV2B, FLSVG) have sialic acid levels which are again similar to parent cell lines. Hamster cell lines and their transformants give a similar correlation. As was observed in experiments measuring sialic acid transferase levels, Py 3T3 has sialic acid levels that are similar to normal cells and does not exhibit the alteration which in other cell lines is associated with transformation.

A striking correlation is found between sialic acid levels and the sialic acid transferase levels in extracts from the various cell lines (Figure 3). From Figure 3, it is intriguing to speculate that at least part of the control of sugar levels in glycoproteins arises from glycosyltransferase levels.

Treating reaction products formed from the reaction of CMP-[^{14}C]sialic acid with desialized fetuin and bovine submaxillary mucin with either neuraminidase (*Vibrio cholerae*) or acid hydrolysis at 80° for 1 hr in 0.1 N H_2SO_4 resulted in quantitative release of radioactivity as sialic acid. Acid hydrolysis of the product formed from incubating UDP-[^{14}C]galactose and desialized β-galactosidase-treated fetuin in 2 N CF_3COOH at 100° for 3 hr quantitatively released galactose.

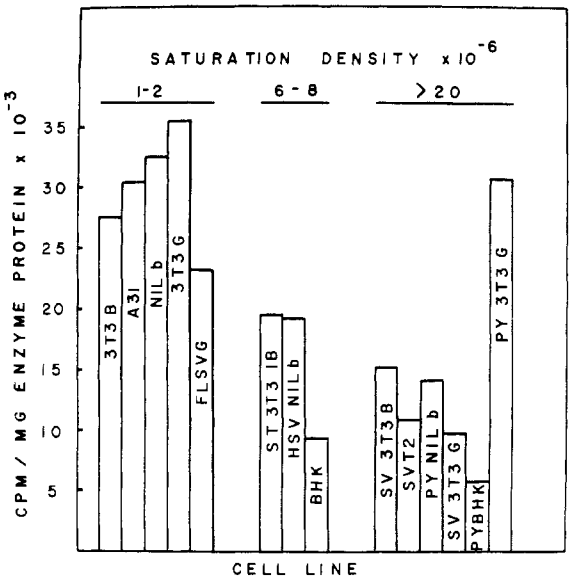

FIGURE 1: Ability of enzyme preparation from normal and transformed cells to catalyze sialic acid transfer to desialized bovine submaxillary mucin. The data are taken from Table I. The enzyme specific activities were measured as described under Materials and Methods and in the legend to Table I. The cells are grouped according to their saturation densities, expressed as the number of cells per 20 cm^2 growing area at confluency as described under Materials and Methods.

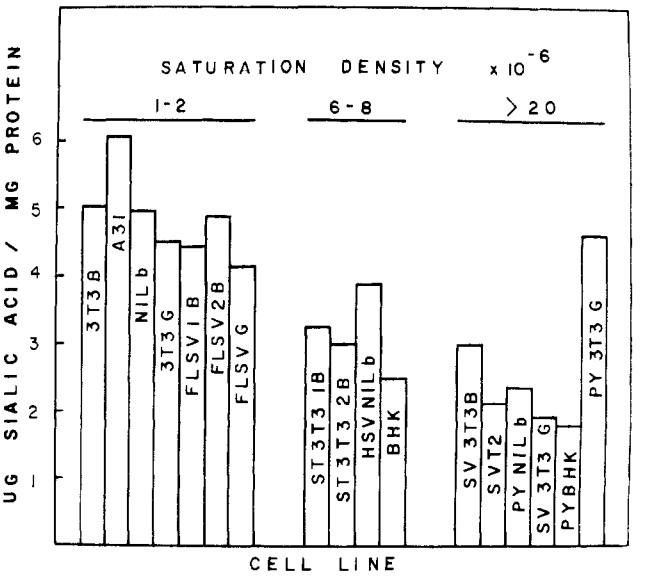

FIGURE 2: Sialic acid levels of normal and transformed cells. The data are taken from Table I. Sialic acid levels were measured as described under Materials and Methods. Cells are divided according to their saturation densities, expressed as cells per 20 cm^2 growing area at confluency.

Sialic acid and galactose containing products which voided when chromatographed through Bio-Gel P-100 were retained by Bio-Gel P-10 after treatment with Pronase.

Bovine submaxillary mucin and fetuin both contain several kinds of oligosaccharide side chains which could serve as acceptors in glycosyltransferase reactions. Previously, we have reported that the product formed when desialized fetuin or bovine submaxillary mucin were reacted with CMP-[^{14}C]sialic acid was alkali labile (Grimes *et al.*, 1971; Grimes and Robbins, 1972). No sialic acid was transferred to the asparagine-linked oligosaccharide of fetuin. All of the radioactivity in [^{14}C]sialic acid containing fetuin and bovine submaxillary mucin was converted to a product with the apparent molecular weight of a trisaccharide by β elimination. To further characterize this product, the following experiments were performed.

[^{14}C]Sialic acid containing fetuin and bovine submaxillary mucin were treated with Pronase and the resulting glycopeptides purified by chromatography through DEAE-cellulose and Bio-Gel P-10. The radiolabeled glycoprotein reaction products were also treated with alkali in the presence of borohydride to produce β-elimination oligosaccharides as described (Bertolini and Pigman, 1970; Grimes and Robbins, 1972). The ^{14}C-labeled oligosaccharides were purified first by chromatography through Bio-Gel P-2 and Dowex 1 (acetate) and then by paper chromatography on Schleicher and Schuell orange label acid-washed paper (Grimes and Robbins, 1972). Chromatograms were developed for 24 hr in ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Radioactive bands were eluted and concentrated prior to further characterization. Purified glycopeptides and oligosaccharides were hydrolyzed in 2 N trifluoroacetic acid for 3 hr at 100° as described (Albersheim *et al.*, 1967). Because of the low levels of oligosaccharide obtained in *in vitro* reactions, the identity of sugar released was determined using NaB^3H_4 reduction. In a typical reaction, hydrolysates were dried and to each was added 40 μl of 0.5 N aqueous NH_3 and 10 μl containing 1 mCi of NaB^3H_4 (New England Nuclear, 250 Ci/mol). After 1 hr at room temperature, excess borohydride was destroyed by adding 1 N acetic acid and the reaction was dried at 40°. MeOH (1 ml) was added to the dried reaction and evaporated to remove excess borate. This was repeated a total of five times. This procedure will label hexoses and hexosamines but not alditols with "^{3}H."

Mixtures of tritium-labeled alditols prepared from hydrolyzed oligosaccharides and glycopeptides were separated into hexosaminitols and neutral sugar alditols by eluting them through ion-exchange columns. The mixtures were passed through micro columns containing 2 ml of Dowex 50 (H^+) and then through micro columns containing 2 ml of Dowex 1 (acetate). The columns were washed with 10 ml of distilled H_2O. Radiolabeled material washing through both columns contains neutral sugar alditols. Radiolabeled material retained on the Dowex 50 column was eluted with 10 ml of normal HCl. This fraction contains the hexosaminitols. The elutions were lyophilized and further characterized by chromatography on paper. Radiolabeled hexosaminitols were resolved by chromatography for 48 hr in a solvent composed of ethanol, methyl ethyl ketone, and 0.5 M morpholine tetraborate as described (Carminatti and Passeron, 1966). This solvent separates galactosaminitol, glucosaminitol, and mannosaminitol. Chromatographing hexosaminitols from the NaB^3H_4 reduced acid hydrolysates of glycopeptides from [^{14}C]sialic acid containing fetuin and bovine submaxillary mucin produced only ^{3}H-labeled galactosaminitol. The β-elimination oligosaccharides (which have a reduced terminal sugar) did not yield any labeled hexosaminitol. The neutral sugar alditol fraction from the hydrolysis of both [^{14}C]sialic acid containing bovine submaxillary mucin and fetuin glycopeptides and β-elimination oligosaccharides was converted to alditol acetates for analysis as described (Albersheim *et al.*, 1967). Alditol acetates were chromatographed on paper which had previously been dipped in a solution composed of 25%

dimethyl sulfoxide in toluene. The chromatograms were developed at 2° for 24 hr in ligroin (bp 66–75°) (Wickberg, 1962). This solvent separates the alditol acetates of mannose, galactose, and glucose. The alditol acetate of galactose was the only labeled sugar detected in NaB^3H_4-reduced hydrolysates of the glycopeptides and β-elimination oligosaccharides. These results are consistent with the following structure for the *in vitro* reaction product formed from the reaction of either desialized fetuin or bovine submaxillary mucin with cellular sialic acid transferase and CMP–sialic acid:

polypeptide-*N*-acetylgalactosamine–galactose–sialic acid

Further evidence supporting the postulated structure came from experiments in which [^{14}C]sialic acid containing fetuin and bovine submaxillary mucin from *in vitro* reactions were treated with alkali in β-elimination reactions in the presence of NaB^3H_4. In a typical reaction, 7600 cpm of [^{14}C]sialic acid containing bovine submaxillary mucin was dried; 50 μl of 0.1 N NaOH and 10 μl of NaB^3H_4 (1 mCi) was added and the reaction was stored for 5 days at room temperature in the dark as described (Grimes and Robbins, 1972). At the end of the incubation, excess borohydride was destroyed by adding dilute acid. The reaction mixture was purified by chromatography first through Dowex 1 (acetate) and then Bio-Gel P-2 as described (Grimes and Robbins, 1972). A peak of dual-labeled material with the apparent molecular weight of a trisaccharide was recovered from the Bio-Gel P-2 column. This peak contained 6000 cpm of ^{14}C and 300,000 cpm of ^{3}H. Hydrolysis with 2 N CF_3COOH yielded only galactosamine and no neutral sugar or glucosamine labeled with ^{3}H.

One problem in the interpretation of these results was that we routinely obtained from 3 to 10 times as much ^{3}H in NaB^3H_4 reductions as we would expect based on the specific activity of the [^{14}C]sialic acid in the *in vitro* product. For example, in the experiment cited above, we obtained 6000 cpm of ^{14}C and 300,000 cpm of ^{3}H. This is a ratio of 50:1. From the specific activity of the [^{14}C]sialic acid and NaB^3H_4, we would expect a ratio of 12.5:1. Thus we obtained four times as much tritium as expected. It is possible that the excess material comes in from the crude cellular extracts used in the *in vitro* reactions. If cells have oligosaccharides on glycoproteins which are similar to the *in vitro* product, then they would be included in the purifications and contribute to the ^{3}H counts. The fact that the sialic acid transferase level and cellular sialic acid levels are correlated would make this a likely possibility. The proposed structure has been reported as a minor oligosaccharide of fetuin (Spiro, 1970).

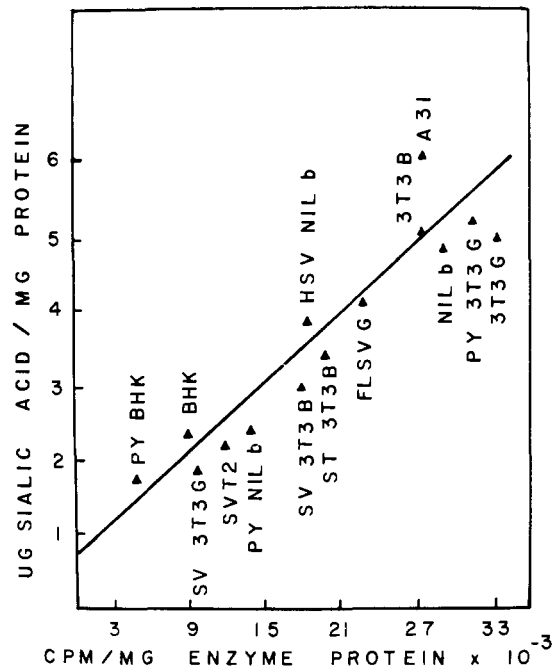

FIGURE 3: Comparison of whole cell sialic acid levels and ability of cell extracts to catalyze sialic acid transfer to desialized bovine submaxillary mucin. The data are summarized from Table I.

Discussion

Sialic acid, galactose, and fucose transferases have been observed in many animal tissues and in cells grown in tissue culture. Ability to catalyze fucose transfer to desialized β-galactosidase-treated fetuin has been observed in extracts from HeLa cells (Bosmann *et al.*, 1968a). Fucosyltransferases have also been observed in porcine liver extracts (Jabbal and Shachter, 1971). Extracts from sheep submaxillary glands will catalyze sialic acid transfer to submaxillary mucins (Carlson *et al.*, 1966). The submaxillary transferase catalyzes sialic acid transfer to *N*-acetylgalactosamine on the polypeptide. Sialic acid transferase from thyroid catalyzes sialic acid transfer to desialized fetuin and thyroglobulin glycopeptides (Spiro and Spiro, 1968a). The sialic acid transferase studied here appears to differ from those enzymes. No transfer of sialic acid to either *N*-acetylgalactosamine of desialized bovine submaxillary mucin or to the asparagine-linked oligosaccharide of fetuin could be detected. The galactosyltransferase in the normal and transformed cell lines is similar to the enzyme catalyzing galactose transfer to fetuin glycopeptides in thyroid glands (Spiro and Spiro, 1968b).

Determining levels of glycosyltransferases in particulate extracts is difficult. A variety of factors could contribute to the observed *in vitro* activity. In BHK cells, ability to catalyze transfer of sialic acid to lactosyl ceramide was enormously stimulated by adding phosphatidylglycerol (Den *et al.*, 1971). The same enzyme activity in polyoma virus transformed BHK cells was only partially stimulated by the same treatment. These and other factors could contribute to measured enzyme differences. In the normal and transformed cell systems studied here, no differences in activities were observed in the presence of phosphatidylglycerol. The reactions have been conducted under incubation conditions where the activity is linear with respect to both time of incubation and enzyme concentration. Incorporations were studied under conditions where there was little or no substrate or product breakdown (except for fucose incorporation). Therefore, it is likely that the activities measured *in vitro* are proportional to enzyme levels.

It has been postulated that control of the carbohydrate structures produced on glycoproteins and glycolipids resides in multiglycosyltransferase systems which are specific both for the sugar transferred and acceptor (Roseman, 1971). The observation that a cellular sialic acid transferase could use both desialized bovine submaxillary mucin and fetuin has important implications for this hypothesis (Grimes *et al.*, 1971). Furthermore, evidence has been published which suggests that the same enzyme can use monosialyl ganglioside as acceptor, producing disialyl ganglioside (Gdla) (Grimes and Robbins, 1972; Grimes *et al.*, 1971). These observations might be interpreted to mean that the transferase has little specificity for an acceptor. The microchemical characterization

reported here shows that the sialic acid transferase is actually highly specific. Only the disaccharide galactose-*N*-acetylgalactosamine is used as acceptor. Monosialyl ganglioside also has galactose-*N*-acetylgalactosamine as terminal sugars, fitting with the observed specificity of the enzyme. Thus, this enzyme falls within the multi-glycosyltransferase system hypothesis, but can apparently use the disaccharide as acceptor independently of the protein (or lipid) that the sugars are attached to.

The enzyme gave a surprising correlation with the total cellular sialic acid level of a cell line. This was interpreted as suggesting a possible role of the enzyme in quantitative as well as qualitative control of glycoprotein synthesis. If the enzyme is in fact responsible for a large proportion of the cellular sialic acid on glycoproteins and glycolipids, then the oligosaccharide characterized here must represent a predominant kind of oligosaccharide found in cells. This possibility can be tested using NaB^3H_4 β elimination on cellular extracts.

The biochemical basis of cellular transformation and cancer must await elucidation of the biochemical structure and organization of the cell surface. Biochemical studies such as the one reported here are only a beginning. Whether sialic acid transferases, glycolipid transferases or other cell surface alterations are primary to transformation is as yet unanswerable. However, transformation by SV40 clearly leads to reduction in levels of sialic acid and sialic acid transferases per milligram of cellular protein in three separate lines of normal mouse cells. Spontaneous transformants also show a partial loss in both parameters. Flat revertants, selected from SV40-transformed cells show a partial recovery of sialic acid and sialic acid transferase levels. Most other cell lines show a marked decrease in sialic acid and sialic acid transferase levels upon transformation. The exception to the rule, Py 3T3, indicates that reduction in these biochemical parameters is not absolutely necessary for loss of density-dependent control of cell division. One possibility is that the gross compositional changes occurring upon SV40 transformation include many secondary changes in addition to the postulated primary cell surface alteration in transformation. Transformation by Py virus in 3T3 cells (but not by Py virus in hamster cells) might cause less secondary change, but still cause the postulated primary cell surface alteration. Another possibility is that transformation of 3T3 cells by Py virus (normally a productive infection) may lead to only a partial alteration of cell surface structures.

It is clear that studies of biochemical events in transformation are complexed by the variety of changes which are observed in glycoproteins, glycolipids, and glycosyltransferases. The fact that many cell surface parameters are changing, likely means that most are secondary to transformation.

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