Sialic Acid Transferases and Sialic Acid Levels in Normal and Transformed Cells*

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ABSTRACT: We have measured the ability of particulate enzyme preparations from Swiss mice 3T3 cells, spontaneously transformed 3T3 cells, and SV40-transformed 3T3 cells to catalyze the transfer of sialic acid to desialized bovine submaxillary mucin and fetuin. Sialic acid content of these cells was also determined. Preparations from transformed cells were found to have only 55–60% of the sialyl transferase activity found in identical preparations from normal cells. The levels of sialyl transferases measured in extracts from spontaneously transformed 3T3 cells were intermediate between the levels of activity in extracts from normal and transformed cells. Measurements of the amounts of sialic acid in whole cell preparations and particulate fractions from 3T3 cells and from SV40-3T3 cells showed that the transformed cells had only 60% as much sialic acid as did normal cells. Similar results were obtained in experiments with Balb/c cells. Balb/c cells which had been transformed by SV40 were found to have only 31% of the sialyl transferase activity found in identical preparations from nontransformed Balb/c cells. Measurements of the amounts of sialic acid in whole cell preparations showed that the transformed cells had only 35% as much sialic acid as did normal cells.

Cells transformed by oncogenic viruses are capable of growth even at high cell densities. Normal cells divide at a rate equal to that of transformed cells at low cell densities but are incapable of cell division once they have contacted other cells. This contact inhibition or density-dependent inhibition of cell division is believed to result from interactions of cell surfaces. Alterations of cell surface glycoproteins and glycolipids leading to loss of contact inhibition may play a primary role in oncogenic virus transformation.

Biochemical evidence for alterations in transformed cell surface polymers have been provided from several laboratories. Ohta *et al.* (1968) reported that transformed cells have reduced amounts of sialic acid compared to normal cells from which they were derived. Wu *et al.* (1969) compared the relative amounts of *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acid in normal and transformed cells grown in medium containing radiolabeled glucosamine. In those studies, analysis of membrane fractions showed much lower relative content of *N*-acetylgalactosamine and sialic acid and a reciprocal increase in *N*-acetylglucosamine in SV40-3T3 cells as compared to 3T3 cells. In addition, measurements of absolute amounts of neutral and amino sugars present in particulate fractions of 3T3 and SV40-3T3 cells revealed a marked decrease in both neutral and amino sugars per milligram of protein in the transformed cells. Hakomori and Murakami (1968) and Mora *et al.* (1969) analyzed glycolipids in normal and transformed cells. Their experiments indicate that normal cells have higher proportions of complex glycolipids than do transformed cells. Thus, the available evidence leads one to conclude that changes in cell surface polymers do occur upon transformation and that these changes generally result in reduction of carbohydrates of glycoproteins and glycolipids.

Cellular control of synthesis of glycosyl polymers is not well understood. It is believed that sugar sequences and bond linkages of carbohydrates in glycoproteins are controlled by groups of enzymes, each catalyzing the transfer of a particular sugar to a specific site on an acceptor (Roseman, 1966). There is no evidence to indicate whether the overall amount of a particular sugar in glycoproteins or glycolipids is controlled by the amount of a particular transferase specific for that sugar.

Bosmann *et al.* (1968) studied two fucosyl transferases, a galactosyl transferase and a *N*-acetylgalactosaminyl transferase from 3T3 and SV40-3T3 cells. They concluded that transformed cells have higher levels of glycosyl transferases involved in cell surface polymer synthesis than do normal cells. These results would appear to conflict with those from chemical studies of cell surfaces.

In this study, the ability to catalyze sialic acid and fucose transfer to glycoproteins was determined in particulate enzyme preparations from normal and transformed Swiss mouse and Balb/c mouse cells. In addition, quantitative measurements of sialic acid concentrations were made on either whole cell or particulate cell preparations from normal and transformed cells. By comparing sialic acid compositions to activities of sialyl transferases in preparations from normal and transformed cells one can determine whether membrane changes result from alterations of glycosyl polymer synthesizing enzymes during viral transformation. Indeed, such viral modification of synthesis of host glycosyl polymers has previously been demonstrated. In *Salmonella anatum* infected by ϵ phage, alterations in the carbohydrate portion of O-specific lipopolysaccharides are accomplished by repression of host enzymes, synthesis of inhibitors of host cell enzymes, and by production of phage-specific enzymes (Robbins *et al.*, 1965; Bray and Robbins, 1967; Losick and Robbins, 1967; Losick, 1969).

Materials and Methods

The nitrogen pressure homogenizer was obtained from Artesian Metal Industries Inc., Waltham, Mass. [^{3}H]Acetic

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anhydride and L-[^{3}H]fucose were purchased from New England Nuclear Corp., Boston, Mass. Bovine and ovine submaxillary glands were obtained from Pentex Corporation, Kankakee, Ill.

Preparation of GDP-[^{3}H]Fucose. Bekesi and Winzler (1967) have observed that L-fucose, upon injection into a rat, rapidly accumulates in liver in the form of GDP-fucose. This observation has been utilized for preparing radiolabeled GDP-fucose. L-[^{3}H]Fucose (2 mCi, specific activity 4 Ci/mmole) in 0.9% NaCl was injected intraperitoneally into an adult albino rat. After 30 min, the animal was anethesized and killed by decapitation. The liver was immediately removed, cut into thin sections, and dropped into 50 ml of boiling 50% ethanol. After 1 min, the suspension of liver tissue was ground with the aid of a mortor and pestle. The homogenate was boiled for 30 sec and filtered through a layer of Celite. The filtrate was evaporated under nitrogen at 40° to a small volume and suspended in 20 ml of H_2O. The solution was frozen and lyophilized. Approximately 5% of the label initially injected into the rat was recovered as GDP-[^{3}H]fucose. GDP-[^{3}H]fucose was purified by descending paper chromatography in a solvent consisting of 1 M ammonium acetate–ethanol (pH 7.5, 30:75, v/v). The product was characterized by treatment with venom phosphodiesterase which released only [^{3}H]fucose-1-PO_4, and by acid hydrolysis which gave only free [^{3}H]fucose. The extent to which the specific activity of [^{3}H]fucose was diluted by unlabeled fucose from the rat liver was not determined.

CMP-[^{3}H]Sialic Acid. [^{3}H]*N*-Acetylmannosamine was prepared by the method of Brunetti *et al.* (1962) from mannosamine and [^{3}H]acetic anhydride (specific activity 50 mCi/mmole). Following purification, [^{3}H]*N*-acetylmannosamine was converted into [^{3}H]*N*-acetylneuraminic acid by the method of Warren (1959) utilizing enzymes prepared from rat liver. [^{3}H]*N*-Acetylneuraminic acid was purified by passing the reaction through a 2 × 20 cm column of Dowex 1-X8 (formate) (200–400 mesh). The column was eluted with a linear gradient of H_2O and 0.5 N formic acid. [^{3}H]*N*-Acetylneuraminic acid in fractions eluting from the column was located by the distribution of radioactivity and by color formation in the thiobarbituric acid assay for sialic acid (Warren, 1959).

CMP-sialic acid synthetase was purified from sheep submaxillary glands by a modification of the procedure used by Kean and Roseman (1966). The frozen glands (50 g) were cut into 0.1 thin slices. The slices were extracted for 1 hr in 100 ml of M K-PO_4 buffer (pH 7.6). This crude extract was subjected to centrifugation for 1 hr at 20,000*g* followed by filtration through glass wool. The extract was dialyzed for 12 hr against 4 l. of 0.01 M Tris (pH 7.5) and 1% with respect to mercaptoethanol. The dialyzed extract was chromatographed on a 5 × 15 cm column of DEAE-cellulose equilibrated with 0.01 M K-PO_4 (pH 7.5) and containing 1% mercaptoethanol. Elution was continued until the red hemoglobin band was washed from the column. Proteins voided by the column were discarded. The column was next eluted with 0.01 M K-PO_4 buffer (pH 7.5) containing 1% mercaptoethanol and 0.075 M KCl. Fractions eluting from the column containing protein were pooled and solid ammonium sulfate added to 90% of saturation. Precipitated protein was collected by centrifugation and dialyzed overnight against 3 l. of 0.01 M K-PO_4 buffer (pH 7.0). The ability of such preparations to catalyze synthesis of CMP–sialic acid from CTP and *N*-acetylneuraminic acid was determined by the method of Warren and Blacklow (1962). In a typical preparation, the final yield of CMP–sialic acid synthetase was 110 units (where 1 unit equals 1 μmole of CMP-sialic acid synthesized per hr) in 40 ml of buffer.

For preparation of CMP-[^{3}H]sialic acid, the reaction mixture contained 0.2 g of magnesium acetate, 4 ml of 0.5 M Tris-acetate (pH 8.4), 300 μmoles of CTP, 60 units of enzyme, and 20–100 μmoles of [^{3}H]*N*-acetylneuraminic acid in a total volume of 30 ml. After incubation at 37° for 2 hr, an additional 50 units of enzyme and 300 μmoles of CTP were added and the incubation was continued for an additional hour. The final volume was 50 ml. In this reaction, more than 90% of the initial [^{3}H]sialic acid was converted into CMP-[^{3}H]-sialic acid.

CMP-[^{3}H]sialic acid was purified by a method similar to that described by Spiro *et al.* (1968). Protein was precipitated by the addition of 50 ml of cold acetone and the solution was subjected to centrifugation. The supernatant solution was evaporated to dryness on a flash evaporator at 40°. CMP-[^{3}H]sialic acid in the residue was purified by chromatography first in a solvent consisting of 1.0 M ammonium acetate–ethanol (pH 7.5, 30:75, v/v) and second in 95% ethanol. CMP-[^{3}H]sialic acid was located by scanning the paper with a ultraviolet lamp and also by virtue of its radioactivity. The radiolabeled product was eluted from the paper in distilled water and the pH was adjusted to 6.5 with sodium bicarbonate. The volume of the final solution was adjusted to give 100,000 cpm of CMP-[^{3}H]sialic acid in 5 μl of solution.

Cell Cultures. The line of Swiss mouse fibroblasts utilized in the present experiments was obtained from Dr. Paul Black. All studies were carried out with a cloned population of cells. These cells are referred to as 3T3 cells. Control 3T3 cells were always passaged prior to confluency in order to ensure against selection of noncontact inhibited cells. The control 3T3 cells grew to saturation densities of 9 × 10^5 cells/50-mm plastic petri dish and remained at this density when maintained up to 10 days after reaching confluency. Cells in the present experiments were used in the eighth to fifteenth passage.

A subline of 3T3 cells which was less contact inhibited was produced by continually allowing 3T3 cells to become confluent before passage. Saturation densities of 5–6 × 10^6 cells/50-mm petri dish were achieved by the 25 passage level. This line is referred to as spontaneously transformed cells (ST-3T3) and was utilized at the 30–40 passage levels. SV40-transformed 3T3 cells (SV40-3T3) were obtained from Dr. Paul Black. The transformed cells had a changed morphology and grew to saturation densities of approximately 2–3 × 10^7 cells/50-mm plastic plate. SV40-3T3 cells were utilized in the eighth to fifteenth passage levels.

Sensitive tests, performed during the course of these experiments in the laboratory of Dr. Louis Dienes at Massachusetts General Hospital, showed that Swiss SV40-3T3 and ST-3T3 cells were contaminated by mycoplasma. Swiss 3T3 cells were free of mycoplasma. No influence of mycoplasma on growth rates of the cells was observed. There was no indication that contamination of ST-3T3 or SV40-3T3 cells by mycoplasma has any effect on either glycosyl transferase levels or sialic acid levels.

Balb/c cells were obtained from Dr. George Todaro of the National Institutes of Health. Cell line A31 is an established line of Balb/c cells which are highly sensitive to contact

inhibition. A31 cells grew to saturation densities of 1.2×10^6 cells/50-mm plastic petri dish and remained at this density when maintained up to 10 days after reaching confluency. SVT2 cells are A31 cells after transformation by SV40. SVT2 cells reached saturation densities of greater than 3×10^7 cells/50-mm plastic petri dish. Standard test for mycoplasma contamination were negative for Balb/c cells.

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 per 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.

Preparations of Glycoproteins for Use as Acceptors in Glycosyl Transferase Reactions. Bovine submaxillary mucin (BSM)[1] was prepared from bovine submaxillary glands by the method of Tsuiki *et al.* (1961). BSM extracted from the gland was purified by precipitation with cetyltrimethylammonium bromide. The resulting clot was dissolved in buffer and fractionated with increasing concentrations of ethanol. The fraction precipitating at 60–70% ethanol was collected by centrifugation. This final pellet is dissolved in 200 ml of cold water and dialyzed exhaustively against cold water at pH 7.0. The final solution was lyophilized and the product was stored at −20°.

To remove sialic acid, 1 g of purified BSM was dissolved in 100 ml of distilled water and the pH was adjusted to 1.0 by the addition of 4.5 N H_2SO_4. The solution was heated to 85° for 2 hr. Free sialic acid in aliquots removed from the reaction was measured by the thiobarbituric acid assay (Warren, 1959). After hydrolysis, the solution was neutralized by the addition of 0.1 N sodium hydroxide and dialyzed exhaustively against water. The sialic acid acceptor was lyophilized and the dry powder stored at −20°. Desialized BSM is referred to as BSM h.

Purified fetuin was purchased from Grand Island Biological Company, Rock Island, N. Y. Fetuin (1 g) was dissolved in 50 ml of water and the pH was adjusted to 1.0 with acid. The reaction was then heated to 85° for 2 hr. Release of sialic acid was measured as described previously. Following neutralization, exhaustive dialysis against water and lyophilization the final dried powder was stored at −20°. Desialized fetuin prepared in this manner is referred to as Fet h.

Galactose was removed from desialized fetuin by treatment with β-galactosidase from *Escherichia coli*. The chromatographically purified enzyme (Worthington Biochemical Corp., Freehold, N. J.) showed no protease activity when incubated with Azocoll (Calbiochem, Los Angeles, Calif.). In a typical preparation, 100 mg of desialized fetuin was dissolved in 40 ml of 0.01 M potassium phosphate (pH 7.0). β-Galactosidase (2000 units) was added and the mixture was incubated at 37° for 200 hr. The release of free galactose was determined by the anthrone procedure following precipitation of protein with 10% trichloroacetic acid and subsequent removal of trichloroacetic acid by ether extraction. Enzymatic removal of galactose exposes an *N*-acetylglucosamine residue in fetuin. It has previously been shown that Fet e. can serve as a fucosyl acceptor when incubated with GDP–fucose and enzyme extracts from 3T3 cells (Bosmann *et al.*, 1968). The procedure removed about 70% of the theoretical amount of galactose in fetuin. β-Galactosidase was precipitated from the reaction by the addition of perchloric acid to a final concentration of 0.34 N. After removal of β-galactosidase by centrifugation, the supernatant solution was neutralized by the addition of 0.5 N KOH, dialyzed, lyophilized, and the dry powder obtained stored at −20°. Desialized β-galactosidase treated fetuin is referred to as Fete.

Preparation of Particulate Glycosyl Transferases. A monolayer of confluent or subconfluent cells in a Bellco roller bottle was washed with 50 ml of solution A (0.8% NaCl–0.05% KCl–0.001 M $K\text{-}PO_4$, pH 7.4) containing 10^{-3} M EDTA (buffer I). The cells were removed in 25 ml of buffer I with the aid of a rubber policeman. The cell pellet was suspended in a volume of 0.01 M $K\text{-}PO_4$ (pH 6.5) to give 0.6 ml of packed cells/10 ml of buffer. After standing for 10 min, the 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 sitting in ice for 10 min, unbroken nuclei were removed by centrifugation for 10 min at 600*g*. The supernatant solution was removed and the pellet was resuspended in 10 ml of 0.01 M $K\text{-}PO_4$ (pH 6.5) and recentrifuged. The wash and supernatant solution were pooled and centrifuged at 37,500 rpm in a Spinco SW40 rotor for 1 hr. The resulting pellet was suspended by homogenization in 2 ml of 0.01 M $K\text{-}PO_4$ (pH 6.5), containing 10^{-3} M $MgCl_2$ and 0.1% Triton X100 detergent. This suspension is referred to as the particulate enzyme preparation and contained about 8 mg of protein. All steps in the preparation were carried out at 2–4°.

Glycosyl Transferase Assay. The ability of particulate enzyme preparations to catalyze the transfer of [^{3}H]sialic acid or [^{3}H]fucose to glycoprotein acceptors was determined in a manner similar to that described by Bosmann *et al.* (1968). In a typical reaction, 50 μl of enzyme containing 0.2–0.4 mg of protein was placed in a 3-ml conical centrifugation tube along with 5 μl of solution containing 100,000 cpm of either CMP-[^{3}H]sialic acid (1.1×10^{-2} μmole) or GDP-[^{3}H]fucose (6.8×10^{-2} mμmole assuming a specific activity of 4 Ci/mmole). Then 50 μl containing 0.5 mg of the appropriate acceptor dissolved in 0.01 M $K\text{-}PO_4$ (pH 6.5) and containing 10^{-3} M $MgCl_2$ and 0.1% Triton X100 was added. When BSM h. was used as acceptor, 0.5 mg of acceptor had 0.22 μmole of acceptor site as determined by the amount of sialic acid released during acid hydrolysis. Desialized fetuin as acceptor contains 0.14 μmole of acceptor site/0.5 mg of protein. β-Galactosidase-treated fet h. had 0.10 μmole of acceptor site/0.5 mg of protein.

After incubation at 37°, reactions were stopped by addition of 1 ml of cold 0.5 N HCl containing 1% phosphotungstic acid. The contents of the tube were throughly mixed with the aid of a Vortex shaker and subjected to centrifugation. The resulting pellet was washed three times with 1 ml of cold 5% trichloroacetic acid. The washed pellet was suspended in 0.5 ml of absolute ethanol and 0.5 ml of ether added to extract trichloroacetic acid. After removal of the ether–ethanol, the residue was dissolved in 1 ml of normal aqueous ammonia by gentle heating for 2–4 hr. After sitting overnight, the dissolved protein was placed in a scintillation vial along with 10 ml of Patterson–Greene solution (Patterson and

[1] Abbreviation used is: BSM, bovine submaxillary mucin.

TABLE I: Incorporation of Sialic Acid and Fucose into Glycoprotein.[a]

Enzyme	Acceptor	cpm Incorpd Expt I	cpm Incorpd Expt II
	A. Sialic Acid		
SV3T3	None	258	244
SV3T3	BSM h.	5,388	5,079
SV3T3	FET h.	6,087	5,678
SV3T3	BSM	761	743
SV3T3	FET	1,035	1,087
Boiled	FET h.	209	169
3T3	None	326	323
3T3	BSM h.	9,525	8,749
3T3	FET h.	10,278	10,855
3T3	BSM	624	672
3T3	FET	1,034	1,128
Boiled	FET h.	251	246
	B. Fucose		
SV3T3	None	404	344
SV3T3	FET e.	7,601	7,463
3T3	None	421	354
3T3	FET e.	11,352	13,294

[a] Particulate enzyme preparations from 3T3 and SV40-3T3 cells were incubated for 2 hr in duplicate with CMP-[^{3}H]-sialic acid (part A) or GDP-[^{3}H]fucose (part B) and the indicated glycoprotein acceptors. Boiled enzyme was a particulate preparation from either 3T3 or SV40-3T3 cells which had been heated to 100° for 15 min. The acceptor abbreviations are: BSM, native bovine submaxillary mucin; Fet, native fetuin; BSM h., BSM after removal of sialic acid by acid hydrolysis; Fet h., fetuin after removal of sialic acid by acid hydrolysis; Fet e., Fet h. after the enzymic removal of galactose by β-galactosidase.

TABLE II: Effect of Detergent on Glycoprotein Sialyl Transferase.[a]

Enzyme Prepared from SV3T3 Cells		
Detergent (0.1%)	Acceptor	cpm
None	None	222
None	BSM h.	472
Triton X100	BSM h.	1829
SDS[b]	BSM h.	345
DOC[b]	BSM h.	829

[a] The pellet obtained when homogenized SV40-3T3 cells were subjected to centrifugation at 100,000g for 1 hr was suspended in either buffer (0.01 M K-PO_4, pH 6.5) or 0.1% of the indicated detergents in buffer. The enzyme preparation along with acceptor and CMP-[^{3}H]sialic acid was incubated for 2 hr at 37°. [b] SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate.

Greene, 1965) containing 33% Triton X100. Tritium counts were made in a three-channel Packard scintillation spectrometer.

Preparation of Whole Cells and Cell Fractions for Sialic Acid Measurements. The monolayers of confluent or subconfluent cells were washed with 50 ml of solution A containing 100 mg/l. of $MgSO_4$ and $CaCl_2$ (buffer II). The cells were removed from the bottle in 25 ml of buffer II with the aid of a rubber policeman. The cell suspension was subjected to centrifugation at 600g for 5 min. The resulting pellet was washed once by suspension in 25 ml of buffer II and resedimented. For whole cell preparations, the washed pellet was stored frozen until used for sialic acid determinations. For homogenization and cell fractionation 0.6 ml of washed cell pellet was suspended in 10 ml of buffer II. Cells were broken in the nitrogen pressure homogenizer. A nuclear pellet was prepared by subjecting the homogenate to centrifugation at 600g for 10 min. The resulting pellet was washed in 10 ml of buffer II and resedimented. This pellet was designated the nuclear pellet (N). The pooled supernatant solution plus washes were subjected to centrifugation at 20,000g for 15 min. The pellet obtained was designated the mitochondrial pellet (Mit.). The supernatant solution and washes from the mitochondrial pellet were subjected to centrifugation at 100,000g for 1 hr. The pellet from this centrifugation was designated the microsomal pellet (Mic). The pellets were suspended by homogenization into 1 ml of water and stored at −20°. All operations were carried out at 2–4°.

Measurement of Sialic Acid Concentrations. For the determination of sialic acid 0.5 ml of cells or cell fractions were adjusted to pH 1.0 by the addition of 4.5 N H_2SO_4. The tubes were sealed and heated to 85° for 1 hr. These hydrolysis conditions result in maximal release of sialic acid from all membrane preparations. After hydrolysis, duplicate aliquots of 50 μl were removed and assayed for protein by the Lowry procedure (Lowry *et al.*, 1951). Duplicate 0.1-ml aliquots were removed and assayed for sialic acid by a micromodification of the standard thiobarbituric acid assay (Strauss *et al.*, 1970). As little as 0.25 μg of sialic acid in an aliquot could be measured by this procedure. Absorbancies were routinely read at 549 and 532 mμ and the contributions of chromophores absorbing at 532 mμ were eliminated as described by Warren (1959).

Results

Glycoprotein Glycosyl Transferases from 3T3 and SV40-3T3 Cells. Particulate extracts from 3T3 or SV40-3T3 cells catalyzed the transfer of [^{3}H]sialic acid and [^{3}H]fucose from CMP-[^{3}H]sialic acid and GDP-[^{3}H]fucose, respectively, to glycoprotein acceptors (Table I). There was insignificant incorporation into acid-insoluble material when acceptor was left out or when enzyme that had been boiled for 15 min was used. All transferase activities were located in particulate enzyme preparations. Native unhydrolyzed fetuin and bovine submaxillary mucin were poor acceptors. Duplicate assays of incorporation were reproducible with an error that was generally less than 10%.

Incorporation of radioactivity into glycoproteins was stimulated by the addition of 0.1% Triton X100 (Table II).

TABLE III: Recentrifugation of Triton X100 Treated Enzyme.[a]

Enzyme	Acceptor	cpm
SV3T3 control	None	251
SV3T3 control	BSM h.	9,051
SV3T3 pellet	BSM h.	6,900
SV3T3 supernatant	BSM h.	1,989
3T3 control	None	325
3T3 control	BSM h.	10,100
3T3 pellet	BSM h.	5,794
3T3 supernatant	BSM h.	1,818

[a] An aliquot of a particulate enzyme preparation from 3T3 and SV40-3T3 cells was assayed for ability to catalyze transfer of [^{3}H]sialic acid to glycoprotein in the presence and absence of acceptor. The remaining enzyme solution (in 1.8 ml of buffer containing 0.1% Triton X100 was subjected to centrifugation at 100,000*g* for 1 hr. The supernatant solution (1.8 ml) was removed and assayed for ability to catalyze [^{3}H]-sialic acid transfer to glycoprotein. The remaining pellet was suspended in 1.8 ml of 0.1 M buffer that contained 10^{-3} M $MgCl_2$ and 0.1% Triton X100 and an aliquot assayed for ability to catalyze [^{3}H]sialic acid transfer to glycoprotein. Incubations were for 2 hr at 37°.

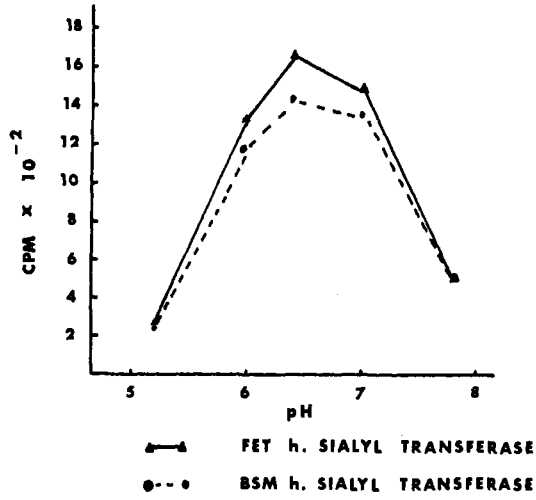

FIGURE 1: The ability of glycosyl transferase preparations from 3T3 cells to catalyze transfer of [^{3}H]sialic acid to BSM h. or Fet h. at several hydrogen ion concentrations. The particulate enzyme preparation was divided into five tubes containing 0.4 ml of enzyme. To each was added 0.3 ml of 0.1 M $K\text{-}PO_4$ buffer at a pH ranging from 5.0 to 8.0. The final pH of each enzyme preparation was then measured. Aliquots were removed and assayed for ability to catalyze sialic acid transfer to glycoprotein. Incubations were for 2 hr at 37°.

Other detergents were not as effective in causing a stimulation of observable enzyme activity. Higher concentrations of Triton X100 approaching 1% caused inhibition of [^{3}H]sialic acid transfer to glycoprotein. From the data of Table III, it is apparent that Triton X100 does not cause a solubilization of the enzyme. Centrifugation of particulate enzyme preparaitons suspended in 0.1% Triton X100 at 100,000*g* for 1 hr. demonstrates that over 75% of the enzyme remains associated with particulate material.

Effect of Hydrogen Ion Concentration on Sialyl Transferases. The optimal hydrogen ion concentration for the enzyme-catalyzed transfer of [^{3}H]sialic acid to glycoprotein acceptors was found to be at pH 6.5 for particulate enzyme preparations from 3T3 (Figure 1) and SV40-3T3 cells (Figure 2). This pH optimum was independent of the desialized glycoprotein used as an acceptor.

Chromatography of Reaction Products. The products of the glycosyl transferase reactions with particulate enzyme preparations from 3T3 and SV40-3T3 cells were characterized by descending paper chromatography. Radioscans of such chromatograms are shown in the data of Figures 3 and 4. Labeled glycoprotein is immobile and remains at the origin. Radioactivity was found at the origin only when reactions were incubated with acceptor. Furthermore, the radioscans reveal that after 2-hr incubation at 37° with enzyme preparations from either normal or transformed cells, a large proportion of the tritium counts were still found as CMP–[^{3}H]sialic acid. Therefore, during the course of the reaction CMP–[^{3}H]sialic acid was always present in excess and was at no time limiting.

The CMP–[^{3}H]sialic acid used in the enzyme reactions contained approximately 30% [^{3}H]sialic acid which resulted from breakdown during storage. After incubation of CMP–[^{3}H]sialic acid with particulate enzyme preparations from either normal or transformed cells the proportion of free [^{3}H]sialic acid was still 30% of the total label. From this result it is evident that there is little or no enzyme activity in either 3T3 or SV40-3T3 extracts which catalyze hydrolysis of [^{3}H]sialic acid from either CMP–[^{3}H]sialic acid or [^{3}H]-sialic acid containing glycoprotein reaction products.

In reactions in which GDP–[^{3}H]fucose was used as substrate, no radioactivity at chromatogram origins was found unless glycoprotein acceptors were added to the reaction mixtures. Free [^{3}H]fucose was found only when glycoprotein acceptor was added. It is likely that the particulate enzyme preparations contain fucosidases that are specific for the

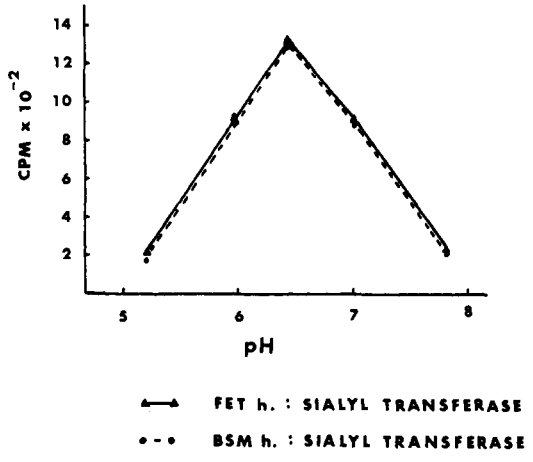

FIGURE 2: The ability of particulate enzyme preparations from SV40-3T3 cells to catalyze transfer of [^{3}H]sialic acid to BSM h. or Fet h. at several hydrogen ion concentrations. Details of the experiment are given in the legend of Figure 1.

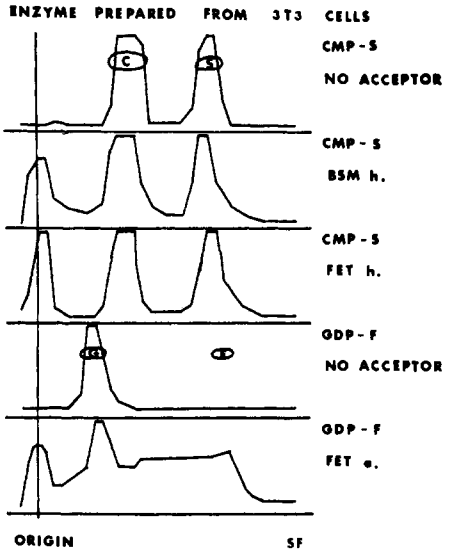

FIGURE 3: Particulate enzyme preparations from 3T3 cells were incubated at 37° for 2 hr in the presence of CMP-[^{3}H]sialic acid (CMP-S) or GDP-[^{3}H]fucose (GDP-F) and with or without acceptor. The reactions were then spotted on the base line of 2 × 22 in. strips of Whatman No. 1 chromatography paper. Strips were subjected to descending chromatography for 16 hr in a solvent consisting of 1 M ammonium acetate–95% ethanol (30:75, v/v). Following chromatography, the strips were scanned for the distribution of radioactivity using a Packard strip-chart scanner. Circled spots indicate the position of migration of authentic standards as follows: (C), CMP-sialic acid; (S), sialic acid; (G), GDP-fucose; (F), fucose.

fucosyl-[^{3}H]glycoprotein bond formed during the reaction. The presence of an active fucosidase in the enzyme preparations makes it difficult to measure absolute activities of fucosyl transferases in either 3T3 or SV40-3T3 cells.

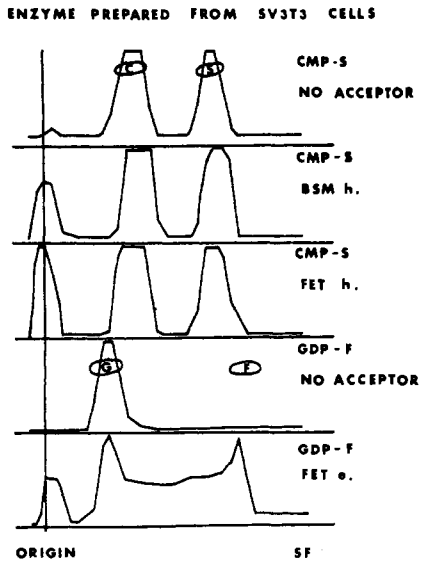

FIGURE 4: Particulate enzyme preparations from SV40-3T3 cells were incubated for 2 hr in the presence of CMP-[^{3}H]sialic acid (CMP-S) or GDP-[^{3}H]fucose (GDP-F) and the indicated acceptors. The details of this experiment are identical with those described in the legend of Figure 3.

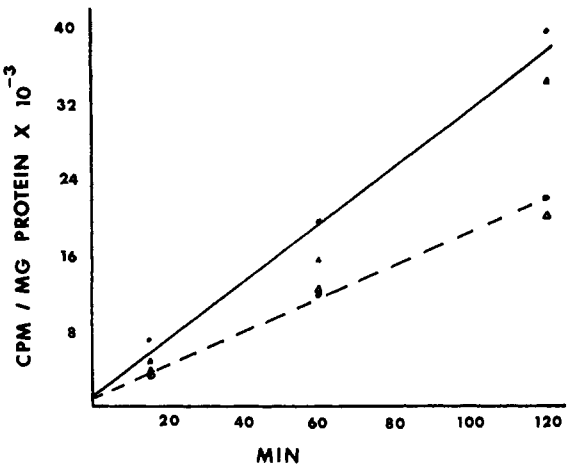

FIGURE 5: Particulate enzyme preparations from 3T3, and SV40-3T3 cells were incubated at 26° in the presence of CMP-[^{3}H]sialic acid and either Fet h. or BSM h. for various times. The specific activity was calculated as counts of [^{3}H]sialic acid transferred to glycoprotein acceptor per milligram of enzyme protein. The solid curve is the activity measured with enzyme preparations from 3T3 cells using Fet h. (●) or BSM h. (▲) as acceptor. The dashed curve is the activity measured with enzyme preparations from SV40-3T3 cells using Fet h. (○) or BSM h. (△) as acceptor.

Absolute Activities of Glycoprotein Glycosyl Transferases from 3T3 and SV40-3T3 Cells. The data in Table IV demonstrate that the specific activities of glycoprotein-glycosyl transferases are constant for preparations of 3T3 or SV40-3T3 cells whether cells were harvested at low or at high cell densities (growing or confluent). Since 3T3 cells at confluency do not divide, it is apparent that the specific activity of the glycosyl transferases does not depend on whether or not cells are actively dividing.

The specific activities of glycosyl transferase preparations were consistently found to be lower in particulate preparations from SV40-3T3 cells than in identical preparations from 3T3 cells. In several experiments, the ability of particulate extracts from SV40-3T3 cells to catalyze [^{3}H]sialic acid transfer to glycoprotein acceptors was only 60% of that found for 3T3 cells when expressed as counts per minute transferred per milligram of protein in 2-hr incubations.

The data of Table V compare the ability of particulate extracts from 3T3 cells, ST-3T3 cells, and SV40-3T3 cells to catalyze sialic acid transfer to desialized fetuin. Regardless of the length of incubation time, the enzyme specific activity found in preparations from ST-3T3 cells was intermediate between that found for 3T3 cells and SV40-3T3 cells. The incorporations were measured at both 15 and 30 min since at 37° the incorporation is not completely linear with respect to time.

A plot of specific activity of sialic acid transferases expressed as counts per minute of [^{3}H]sialic acid incorporated per milligram of protein as a function of the length of incubation is shown in Figure 5. In this experiment, reactions were incubated at 26° rather than at 37°. Under these conditions, the incorporation is linear with respect to time. From the curves of Figure 5 it was calculated that SV40-3T3 cells had a specific activity of sialic acid transferases that was 58% of that found in identical preparations from 3T3 cells. This difference in specific activity is identical to the differences

TABLE IV: A Comparison of Glycoprotein–Glycosyl Transferase Activities in Preparations from 3T3 and SV40-3T3 Cells.[a]

Substrate	Acceptor	Enzyme Source (cpm/mg of Protein per 2 hr) Confl 3T3	Grow 3T3	"Confl" SV3T3	Grow SV3T3
		Part A			
CMP-N	None	496	331	700	383
CMP-N	BSM h.	18,000	18,400	12,000	12,800
CMP-N	FET h.	40,800	36,800	22,700	22,000
GDP-F	None	1,830	2,460	1,420	1,830
GDP-F	FET e.	92,000	81,000	53,000	48,600

Enzyme	Part B cpm/mg of SV3T3 / cpm/mg of 3T3
BSM h. sialyl transferase	0.68
FET h. sialyl transferase	0.58
Fet e. fucosyl transferase	0.59

[a] (A) Particulate enzyme preparations from 3T3 and SV40-3T3 cells were assayed for ability to catalyze [^{3}H]sialic acid or [^{3}H]fucose transfer to glycoprotein acceptors. Enzyme preparations were from growing 3T3 cells (Grow 3T3) which were in a logrithmic phase of growth and also from nondividing 3T3 cells (Confl 3T3) which had been maintained at confluency for 3 days. Grow SV3T3 refers to an cnzyme preparation from SV40-3T3 cells which were harvested at a low cell density. "Confl" SV3T3 cells refers to an enzyme preparation from SV40-3T3 cells which were harvested at a high cell density. Such cells are still in a logrithmic phase of growth. (B) The average specific activity for catalyzing transfer of [^{3}H]sialic acid or [^{3}H]fucose to the individual acceptors was used to calculate the ratios of specific activities between 3T3 and SV403-T3 cells.

TABLE V: Specific Activity of Particulate Enzyme Preparations from SV40-3T3 Cells, ST-3T3 Cells, and 3T3 Cells for Catalyzing Transfer of [^{3}H]Sialic Acid to Desialized Fetuin.[a]

Enzyme	Acceptor	cpm/mg of Protein per 15 min	cpm/mg of Protein per 30 min
3T3	None		1,480
3T3	FET h.	6900 (100)	11,600 (100)
ST3T3	None		553
ST3T3	FET h.	4900 (71)	7,760 (63)
SV3T3	None		1,140
SV3T3	FET h.	3780 (55)	6,200 (53)

[a] Specific activity calculations were made after 15- and 30-min incubation at 37°. The figures enclosed in parentheses indicate the relative activities arbitrarily giving the value of 100 for activity measured in preparations from 3T3 cells.

seen when reactions were incubated at 37°. Thus, from the data of Tables IV and V and in Figure 5, it is apparent that there is a constant and reproducible difference between 3T3, ST-3T3, and SV40-3T3 cells in enzyme specific activities for catalyzing sialic acid transfer to glycoprotein acceptors.

In all transferase reactions, excess acceptor was present at the beginning of all incubations. Addition of new acceptor after 2-hr incubation does not increase the incorporation of [^{3}H]sialic acid into glycoprotein. In one experiment, particulate enzyme preparations from 3T3 and SV40-3T3 cells were incubated with substrate, acceptor, and 100,000g supernatant solutions from both 3T3 and SV40-3T3 cells. The enzyme activity was constant regardless of the presence of the supernatant solutions. There was no indication of any soluble modifiers or inhibitors of enzyme activity in supernatant solutions from either kind of cell.

Concentrations of Sialic Acid in Preparations from Normal and Transformed Cells. A microthiobarbituric acid assay was used to measure sialic acid concentrations in particulate extracts from 3T3, ST-3T3, and SV40-3T3 cells. Results of several of these experiments are shown in the data of Table VI. The fractionation conditions used did not give clean preparations of nuclei, mitochondria, or microsomes. The bulk of the sialic acid and protein representing a wide mixture of membranous materials, sedimented in the fraction labeled Mit. While there were some differences in distribution of sialic acid between fractions in different experiments, the total sialic

TABLE VI: Sialic Acid Concentrations in Particulate Preparations from 3T3 and SV40-3T3 Cells.[a]

Cell	μg of Sialic Acid/mg of Protein N	Mit.	Mic	T	Av	%
SV3T3	3.82	6.04	3.34	4.85		
SV3T3	4.82	8.0	2.72	5.46	5.02	66
SV3T3	4.70	6.20	3.05	4.76		
SV3T3	4.37	5.14	4.85	5.05		
ST3T3	4.42	4.66	6.08	4.82		
ST3T3	3.84	6.78	4.86	5.40	4.99	67
ST3T3	2.40	5.28	5.02	4.75		
3T3	5.60	8.92	6.60	7.80		
3T3	0.175	8.00	7.30	7.0	7.52	100
3T3	5.85	7.30	12.80	7.80		

[a] Particulate cell preparations from 3T3, ST-3T3, and SV40-3T3 cells were assayed for sialic acid concentrations per microgram of protein. Preparations are from cells at different passages and harvested at different times. The average concentration (micrograms per milligram of protein) of sialic acid from cells was calculated from the total (T) particulate sialic acid concentration. The per cent was determined by giving the amount of sialic acid in extracts from 3T3 cells the arbitrary value of 100.

TABLE VII: Sialic Acid Concentrations from 3T3, ST-3T3, and SV40-3T3 Cells.[a]

Cell	μg of Sialic Acid/ mg of Protein	%
3T3	5.35	100
3T3	4.85	
ST3T3	3.24	63.7
ST3T3	3.26	
SV3T3	3.31	60.6
SV3T3	2.86	

[a] Two separate preparations of each cell type were included in the assays. For the relative concentrations, sialic acid concentration in extracts from 3T3 cells was arbitrarily called 100.

TABLE VIII: Specific Activity of Particulate Enzyme Preparations for Catalyzing Transfer of [^{3}H]Sialic Acid to Desialized Fetuin (Fet h.) and Desialized Bovine Submaxillary Mucin (BSM h.), and the Concentrations of Sialic Acid in Whole Cell Preparations of A31 and SVT2 Cells.[a]

	BSM h. Sialyl Transferase (cpm/mg)		Fet h. Sialyl Transferase (cpm/mg)		Sialic Acid
Cell	15 min	30 min	15 min	30 min	(μg/mg)
A31	7880	12,100	6400	8770	6.10
SVT2	2600	3,300	1940	2660	2.16
	D 31%		D 31%		D 35%

[a] Specific activity calculations expressed as counts per minute incorporated into glycoprotein per milligram of enzyme protein were made after 15- and 30-min incubation at 37°. Sialic acid concentrations were calculated as micrograms of sialic acid per milligram of cell protein. The difference (*D*) is expressed as the specific activity or sialic acid concentration from SVT2 cells divided by the value obtained from A31 cells times 100.

acid concentration per milligram of protein, calculated from the total protein and sialic acid of all particulate fractions, was relatively constant. In some cases, extensive degredation of the nuclei occurred during homogenization. In these cases, little sialic acid or protein was found in the nuclear fraction (N). This was the case in the second experiment using 3T3 cells, where 0.75 μg of sialic acid/mg of protein was found. This fraction contained very little protein and has little effect on the calculation of total sialic acid per milligram of protein for all particulate fractions. The total sialic acid concentration per mg of protein was consistently found to be lower in particulate fractions from SV40-3T3 and ST-3T3 cells as compared with 3T3 cells. The particulate fractions contained over 80% of the total sialic acid of the cell.

Sialic acid concentrations were also measured in whole cell preparations. A summary of the data obtained from these experiments is shown in Table VII. Again, the sialic acid concentrations were significantly lower in preparations of ST-3T3 cells and SV40-3T3 cells as compared to 3T3 cells. The concentrations of sialic acid measured in these experiments were independent of the growth conditions of the cells or whether or not the cells were confluent.

From the data of several experiments, the average sialic acid concentration in preparations from SV40-3T3 cells was found to be about 60% of that found for 3T3 cells.

Sialic Acid Concentrations and Sialyl Transferase Specific Activities from Balb/c Cells. The ability of enzyme preparations from SVT2 cells and A31 cells to catalyze sialic acid transfer to glycoproteins is shown in the data of Table VIII. Regardless of whether BSM h. or Fet h. were used as acceptor and whether reactions were incubated for 15 or 30 min, the sialyl transferase specific activities of preparations from SVT2 cells were 31% of that found for A31 cells. Measurements of sialic acid concentrations of whole cell preparations showed that SVT2 cells had 35% as much sialic acid as did A31 cells.

Discussion

Sialic acid transferases which have been studied will catalyze transfer of sialic acid to either galactose or *N*-acetylgalactosamine on glycoproteins, glycolipids, or oligosaccharides (Spiro, 1969). Enzymes isolated from sheep submaxillary glands are capable of catalyzing transfer of sialic acid only to protein bound *N*-acetylgalactosamine in desialized ovine submaxillary mucin (Carlson *et al.*, 1964). Sialyl transferases isolated from the soluble fraction of Colostrum (Bartholomew *et al.*, 1964) from mammary glands (Jourdian *et al.*, 1963) or from thyroid glands (Spiro and Spiro, 1968) utilize only those acceptors having terminal galactose units. Particulate extracts from 3T3 cells or from SV40-transformed 3T3 cells are capable of catalyzing transfer of sialic acid to glycoprotein acceptors with terminal galactose and terminal *N*-acetylgalactosamine. Similarities in pH optimum for transfer of sialic acid to the two acceptors may indicate that a single enzyme is involved in these reactions.

In this study, it was found that ability of particulate enzyme preparations from SV40-3T3 cells to catalyze sialic acid transfer to glycoproteins was only about 55–60% of that found in identical extracts from 3T3 cells. In addition, the activity levels in extracts from ST-3T3 cells were intermediate between those found in similar extracts from 3T3 and SV40-3T3 cells. Thus, the evidence indicates an inverse correlation between ability of cells to grow at high cell densities and activities of sialic acid transferases in cellular particulate extracts.

Absolute determinations of amounts of total enzyme activities of a cell line is difficult. However, measurements of enzyme activity under conditions where substrate and acceptor are maintained in excess, the reproducibility of such measurements, and the lack of any product breakdown during the reaction indicate that values for enzyme activities should be a real indication of the ability of the cells to catalyze sialic acid transfer. When reactions were incubated at 37°, the rate of incorporation of sialic acid into glycoprotein decreased with time. However, the differences observed in sialic acid

transferase activities were constant, regardless of the incubation time. When incubations were carried out at 26°, the incorporation of [^{3}H]sialic acid into glycoprotein was linear with respect to time. Similar differences between specific activities of sialic acid transferases from normal and transformed cells were observed whether reactions were incubated at 26° or 37°. It is likely that the sialyl transferases are unstable at 37° *in vitro*.

Preliminary studies of the properties of the sialic acid transferases from transformed cells did not give evidence for any new enzymes. However, more extensive studies will be required to prove that the sialyl transferases from normal and transformed cells are the same. No modifiers or inhibitors of sialic acid transferases could be found in supernatant solutions from either 3T3 or SV40-3T3 cells. The most likely explanation for results reported in this communication is that there is a reduction in the amount of the sialic acid transferases per mg of protein when normal 3T3 cells are transformed by SV40 virus. It is likely that the change represents a decreased ability of transformed cells to catalyze sialic acid transfer. However, an apparent decrease in enzyme activity in SV40-3T3 cell preparations would also result if there was an increase in particulate protein in crude extracts of SV40-3T3 cells as compared with 3T3 cells. It is not yet possible to distinguish between these possibilities.

The activity of a fucosyl transferase was also found to be reduced in transformed cell preparations. It may be that many other glycosyl transferases will be found to be reduced in transformed cells. Wu *et al.* (1969) have already reported that many sugars on glycosyl polymers were reduced in concentration in SV40-3T3 cells. However, since there is an active fucosidase in particulate enzyme preparations, measurements of levels of fucosyl transferases are uncertain. No attempt was made to measure fucosidase activities in 3T3 and SV40-3T3 cells.

The report by Bosmann *et al.* (1968) that glycosyl transferases were from two- to fourfold more active in preparations from transformed cells than in similar preparations from normal cells conflicts with the results reported here. While this is the first report of measurements of sialic acid transferases in 3T3 cells the fucosyl transferase which was assayed in these experiments should be identical with the fucosyl transferase which Bosmann *et al.* found to be increased in activity in transformed cells. The discrepancies may be due to the use of different methods for enzyme preparation or may be a result of the activity of the fucosidase which we have observed in our preparations. Another possibility is that different clones of transformed cells, even those transformed by the same virus, will not all show the same changes. The measured decreases in transferase levels reported in this communication are more consistent with the chemical evidence that there is a reduction in polymer bound carbohydrate in transformed cells (Ohta *et al.*, 1968; Wu *et al.*, 1969; Hakomori and Murakami, 1968; Mora *et al.*, 1969).

Quantitative measurements of sialic acid concentrations in particulate fractions and whole 3T3 and SV40-3T3 cells confirm observations of Ohta *et al.* (1968) and Wu *et al.* (1969) that transformed cells have reduced amounts of sialic acid. It is of particular interest that the amount of change which was observed for sialic acid concentration between 3T3 and SV40-3T3 cells (60%) was almost exactly equaled by the change in sialyl transferase activities. This might be an indication that glycosyl transferases determine the amounts of sugars on glycoproteins and glycolipids in addition to their postulated role of determining bond linkages and sequences of sugars. However, the measurements of sialyl transferase levels using desialized BSM or fetuin as acceptors does not necessarily measure all the sialyl transferases in the cell.

Results of experiments with Balb/c normal and transformed cells were similar to those from Swiss mice cell lines. Sialyl transferases in preparations from SVT2 cells were only 31% as active as identical preparations from A31 cells. In addition, sialic acid concentrations of whole SVT2 cells were 35% of that found for A31 cells. Thus, in the Balb/c-transformed cells, as was found for Swiss mice cells, the activity of sialyl transferases and sialic acid concentrations were both found to decrease by a similar amount.

It is difficult to determine whether changes found in transformed cells are of primary or secondary importance in oncogenesis. The inverse correlation between levels of sialyl transferase activities and the saturation density of cells is consistent with a hypothesis that reduction in levels of glycosyl transferases are of primary importance in transformation. It would be of interest to see if revertants (Pollack *et al.*, 1968) of transformed cells which have regained the ability to be contact inhibited have levels of sialyl transferases and sialic acid concentrations that are similar to normal cells. A fundamental question, still unanswered, is whether reductions of carbohydrates of cell surface glycoproteins and glycolipids and reduced transferase activities occur in all cells transformed by SV40. It will also be of interest to see if similar changes occur on cells transformed by other oncogenic DNA viruses and also in cells transformed by RNA viruses.

References

Bartholomew, B., Jourdian, G. W., and Roseman, S. (1964), *Abstracts 6th Int. Congr. Biochem., IUB., 32*, 503.

Bekesi, J. G., and Winzler, R. J. (1967), *J. Biol. Chem. 242*, 3873.

Bosmann, H. B., Hagopian, A., and Eylar, E. H. (1968), *J. Cell Physiol. 72*, 81.

Bray, D., and Robbins, P. W. (1967), *J. Mol. Biol. 30*, 457.

Brunetti, P., Jourdian, G. W., and Roseman, S. (1962), *J. Biol. Chem. 237*, 2447.

Carlson, D. M., McGuire, E. J., Jourdian, G. W., and Roseman, S. (1964), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 23*, 380.

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

Jourdian, G. W., Carlson, D. M., and Roseman, S. (1963), *Biochem. Biophys. Res. Commun. 10*, 352.

Kean, E. L., and Roseman, S. (1966), *Methods Enzymol. 8*, 208.

Losick, R. (1969), *J. Mol. Biol. 42*, 237.

Losick, R., and Robbins, P. W. (1967), *J. Mol. Biol. 30*, 445.

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

Mora, P. T., Brady, R. O., Bradley, R. M., and McFarland, V. W. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 1290.

Ohta, N., Pardee, A. B., McAuslan, B. R., and Burger, M. M. (1968), *Biochim. Biophys. Acta 158*, 89.

Patterson, M. S., and Greene, R. C. (1965), *Anal. Chem. 37*, 854.
Pollack, R. E., Green, H., and Todaro, G. J. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 126.
Robbins, P. W., Keller, J. M., Wright, A., and Bernstein, R. L. (1965), *J. Biol. Chem. 240*, 384.
Roseman, S. (1966), *in* Biochemistry of Glycoproteins and Related Substances, Rossi, E., and Stoll, E., Ed., Basal, S. Karger, 244.
Spiro, M. J., and Spiro, R. G. (1968), *J. Biol. Chem. 243*, 6520.
Spiro, R. G. (1969), *N. E. J. Med. 281*, 991, 1043.
Strauss, J. H., Burge, B. W., and Darnell, J. E. (1970), *J. Mol. Biol. 47*, 437.
Tsuiki, S., Hashimoto, Y., and Pigman, W. (1961), *J. Biol. Chem. 236*, 2172.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Warren, L., Blacklow, R. S. (1962), *J. Biol. Chem. 237*, 3527.
Warren, L., and Glick, M. C. (1966), *Methods Enzymol. 8*, 131.
Wu, H. C., Meezan, E., Black, P. H., and Robbins, P. W. (1969), *Biochemistry 8*, 2509.

Limited Digestion of Citraconylated Bovine Serum Albumin with α-Chymotrypsin*

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ABSTRACT: Citraconyl bovine serum albumin was digested with α-chymotrypsin until eight to nine amide bonds had been broken per protein molecule. Under the conditions of the digestion (*i.e.*, pH 7.7, water solution, 23°) the citraconylated protein, which had nearly 70% of its ε-amino groups modified by citraconic anhydride, existed in an expanded conformation very similar to that of serum albumin in acid (Jonas, A., and Weber, G. (1970), *Biochemistry 9*, 4729). The digest was fractionated on ion-exchange and gel filtration columns, into a small peptide fraction and several macromolecular fragments. These chymotryptic fractions were subsequently studied as to their average molecular weights, rotational relaxation times, amino acid composition, fluorescence spectra, and anion binding properties. Molecular weights and rotational relaxation times for the five macromolecular fractions ranged from 35,000 to 8000 and from 33 to 6 nsec, respectively. The per cent amino acid compositions deviated significantly in polar residue content from that of bovine serum albumin; three of the larger fragments were negatively charged with respect to the intact protein. The small peptide fraction had an average molecular weight near 2000 and was basic relative to the parent protein, due mainly to a high content of arginine residues. At least one of the tryptophan residues was found in this fraction. The isolated fractions had low binding affinities for 1-anilinonaphthalene-8-sulfonate, but the total digest, especially in the presence of excess small peptide fraction, had an increased affinity for the ligand. The results suggest that 50–60% of the expanded bovine serum albumin is relatively compact at the carboxyl end, while the amino-terminal region is loosely organized and is easily broken down by proteases. The experiments also indicate that bovine serum albumin has regions of very uneven charge distribution. The "middle" of the protein appears to be more negative, and the adjacent zones appear to be more basic than the total bovine serum albumin. The anion binding experiments suggest that the small peptide fraction originates at or very near the regions of bovine serum albumin where strong anion binding takes place.

The reversible changes in the physicochemical properties of BSA,[1] in the pH region from 4 to 2, led Harrington *et al.* (1956) and Foster (1970) to propose three-dimensional models of the protein, which consisted of glubular regions joined by flexible polypeptide chains. Based on these models, limited protease digestion experiments were performed on expanded serum albumin, under the assumption that proteases would preferentially attack the extended polypeptide chains liberating a few compact macromolecular fragments.

After a short pepsin digestion of BSA at pH 3.0, Weber and Young (1964a,b) recovered two types of macromolecular fragments having average molecular weights of 32,000 and 12,500. A more prolonged digestion of BSA with pepsin, under similar conditions to those used by Weber and Young (1964a,b), gave several heterogeneous fractions. Purification of these fractions yielded two small, electrophoretically pure segments: the *Phe* fragment (mol wt 8500) from the carboxyl-terminal end, and the *Asp* fragment (mol wt 2800) from the amino-terminal end of BSA (Peters and Hawn, 1967).

Adkins and Foster (1965, 1966) and Pederson and Foster (1969) used subtilisin, in the presence of 100 moles of sodium

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[1] Abbreviations used are: BSA, bovine serum albumin; DNS, 1-dimethylaminonaphthalene-5-sulfonyl fluorescent label; BSA-DNS, BSA labeled with DNS; ANS, 1-anilinonaphthalene-8-sulfonate.