Cushman, D. W., and Cheung, H. S. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, Abstr. 3019.
Cushman, D. W., and Cheung, H. S. (1971), *Biochem. Pharmacol. 20*, 1637.
David, I. B., French, T. C., and Buchanan, J. M. (1963), *J. Biol. Chem. 238*, 2178.
Engel, S. L., Schaeffer, T. R., Gold, B., and Rubin, B. (1971), *Proc. Int. Union Physiol. Sci. XXV Int. Congr., Munich* (in press).
Ferreira, S. H. (1965), *Brit. J. Pharmacol. 24*, 163.
Ferreira, S. H., Bartelt, D. C., and Greene, L. J. (1970a), *Biochemistry 9*, 2583.
Ferreira, S. H., Greene, L. J., Alabaster, V. A., Bakhle, Y. S., and Vane, J. R. (1970b), *Nature (London) 225*, 379.
Gray, W. R. (1967), *Methods Enzymol. 11*, 469.
Greene, L. J., Stewart, J. M., and Ferreira, S. H. (1970), *Advan. Exp. Med. Biol. 8*, 81.
Kato, H., Iwanaga, S., and Suzuki, T. (1966), *Experientia 22*, 49.
Kato, H., and Suzuki, T. (1969), *Experientia 25*, 694.
Kato, H., and Suzuki, T. (1970), *Proc. Jap. Acad. Sci. 46*, 176.
Kimura, T., Kato, H., Sakakibara, S. and Suzuki, T. (1970), *Proc. Amer. Peptide Symp., 2nd, 1970* (in press).
Merrifield, R. B. (1969), *Advan. Enzymol. 32*, 221.
Morris, L., and Piez, K. A. (1960), *Anal. Biochem. 1*, 189.
Ng, K. K. F., and Vane, J. R. (1970), *Nature (London) 225*, 1142.
Ramachandran, L. K., and Witkop, B. (1967), *Methods Enzymol. 11*, 283.
Schroeder, W. A. (1967), *Methods Enzymol. 11*, 351.
Shaltiel, S., and Patchornik, A. (1963), *J. Amer. Chem. Soc. 85*, 2799.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stewart, J. M., Ferreira, S. H., and Greene, L. J. (1971), *Biochem. Pharmacol. 20*, 1557.
Stewart, J. M., and Young, J. D. (1969), Solid Phase Peptide Synthesis, San Francisco, Calif., W. H. Freeman.
Werum, L. N., Gordon, H. T., and Thornburg, W. (1960), *J. Chromatogr. Sci. 3*, 125.
Wolff, F. J. (1969), Separation Methods in Organic Chemistry and Biochemistry, New York, N. Y., Academic Press, p 220.

Gas Chromatographic Determination of Gangliosides in Mouse Cell Lines and in Virally Transformed Derivative Lines*

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ABSTRACT: The distribution of gangliosides in established mouse cell lines in tissue culture was investigated, before and after transformation of the cells with SV40, a tumorigenic DNA virus. A suitable chemical derivatization and gas–liquid chromatography procedure was developed for the carbohydrate residues of the gangliosides. Epithelial-like cell lines from A AL/N strain mouse and highly contact-inhibited fibroblastic 3T3 cell lines from both Swiss and Balb c strain mouse had drastically reduced content of the disialotetrasaccharide ganglioside (G_{D1a}) after the SV40 virus induced transformation in culture. This finding is in complete agreement with our previous observation on these and similar cell lines employing thin-layer chromatography and colorimetric techniques. The method of derivatization and of gas–liquid chromatography for the carbohydrate residues of the gangliosides allowed definitive identification of the mouse gangliosides, and represents an accurate and internally consistent method suitable for the quantitation of the small amount of various gangliosides present in cell lines.

We have reported that when established mouse cell lines in culture are transformed by the tumorigenic DNA viruses polyoma or SV40, a change occurs in the various gangliosides[1] present in the cells. The amount of the higher gangliosides, especially the disialotetrasaccharide ganglioside G_{D1a}, is drastically reduced in the virally transformed cells (Mora *et al.*, 1969; Brady and Mora, 1970). The ganglioside analysis was carried out on the glycolipid extracts after thin-layer chromatography by standard colorimetric methods.

Higher gangliosides, including G_{D1a} and G_{M1}, were also detected by thin-layer chromatography in various other cultivated cells from different species, including in human fibroblasts (Hakomori, 1970), adult hamster kidney epitheloid cells, monkey kidney, etc. (Klenk and Choppin, 1970), and also in two types of mouse cells (Yogeeswaran *et al.*, 1970), but are apparently absent in a particular baby hamster kidney fibroblast cell line (BHK21) previously employed to study

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[1] The nomenclature used for gangliosides is from Svennerholm (1963); G_{D1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl[*N*-acetylneuraminyl]galactosylglucosylceramide; G_{M3}, galactosyl-*N*-acetylgalactosaminyl-[*N*-acetylneuraminyl]galactosylglucosylceramide; G_{M2}, *N* - acetylgalactosaminyl - [*N* - acetylneuraminyl]galactosylglucosylceramide; hematoside, G_{M3}NAc or G_{M3}NG, *N*-acetyl- or *N*-glycolylneuraminylgalactosylglucosylceramide, respectively.

glycolipid changes after virus-induced transformation (Hakomori and Murakami, 1968).

Soon it became evident that it would be advisable to employ a direct and more precise chemical method and to unambiguously identify the carbohydrate residues in the various gangliosides. The carbohydrate residues glucose, galactose, *N*-acetylgalactosamine, *N*-acetyl- (and *N*-glycolyl)neuraminic acid make up the carbohydrate chains in a given ratio and in a certain sequence in the different gangliosides.[1]

Gas–liquid chromatography of the carbohydrates can be used for identification and for quantitation of the gangliosides present in cultivated cells. Recently the tissue culture grown mouse L cells and their isolated surface membranes were analyzed by gas–liquid chromatography for glycolipids including the gangliosides, after carefully separating subfractions containing neutral sugars, amino sugars, and the neuraminic acids (Weinstein *et al.*, 1970). Different appropriate derivatization of the sugars were employed on the subfractions, necessitating separate and different gas–liquid chromatography experiments.

We have chosen to employ an approach, whereby all the monosaccharide residues present in gangliosides can be identified and quantitated simultaneously (Clamp *et al.*, 1967). The method includes conventional lipid extraction procedures and the separation of gangliosides by thin-layer chromatography as described by us earlier (Mora *et al.*, 1969), followed by a common sequence of chemical treatment for the recovered gangliosides which allows separation and quantitation of all the carbohydrate residues present in gangliosides by a single gas–liquid chromatography run. This method thus allows a simpler and internally more consistent quantitation of the small amount of individual gangliosides present in the different cell lines.

This study presents details of this method as developed by us, and data on quantitation of the gangliosides in three tissue culture grown established mouse cell lines and in the SV40 virus transformed respective derivative lines.

Experimental Section

Cell Lines. Three cell lines were chosen, each from a different strain of mouse as representative examples of established mouse cell lines in tissue culture. The origin of the cell lines and their properties were described before (Mora *et al.*, 1969; Brady and Mora, 1970). In short, T AL/N is a "spontaneously" transformed tumorigenic epithelial-like cell line from A mouse. Swiss 3T3 is a highly "contact-inhibited" fibroblast cell line from a Swiss mouse embryo. Balb 3T3 is a similar line from a Balb c mouse embryo. Their SV40 virus transformed derivatives are, respectively, SVS AL/N, Swiss SV 3T3 101, and Balb SV T2. The virus-transformed cells are clonal derivatives of single cells; they continue to carry SV40 T antigens, and also tumor-specific transplantation antigens detectable by virus-specific cytotoxic antiserum (Smith *et al.*, 1970); however, they do not produce infectious virus. The virus transformed cells all grow to a higher "saturation density" in culture than the cells from which they were derived.

Cell Growth and Harvesting Conditions. All cells were grown in 1-l. glass roller bottles in Dulbecco-Vogt modification of Eagle's medium with 10% fetal calf serum. Cells were harvested by saline when in their logarithmic phase of growth before reaching confluency, as described previously (Brady and Mora, 1970). When not immediately analyzed the cells were stored at −70° under minimum amount of saline.

Glycolipid Extraction and Thin-Layer Chromatographic Separation of Gangliosides. The washed packed cells (1–2 ml, 70–160 mg of protein) were brought into a homogeneous suspension by thorough agitation with a Vortex mixer in 30 ml of ice-cold saline. Protein was determined in a 10-μl aliquot by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as standard.

The whole amount of cell suspension was centrifuged (*ca.* 5 min at 700*g*) and the packed cells were suspended in three volumes of 0.01 M KCl and 20 volumes of $CHCl_3$–CH_3OH (2:1, v/v). The cells were homogenized with three to four strokes in a tight-fitting small (40-ml) Dounce homogenizer and the mixture was heated for 15 min at 50°. The cell material was removed by filtration, suspended in ten volumes of $CHCl_3$–CH_3OH (1:2, v/v), again homogenized, and heated for another 15 min at 50°. The denatured protein was removed by filtration. The second extract was brought to dryness in a warm water bath (*ca.* 40°) under a stream of nitrogen in a graduated centrifuge tube. Into the tube containing the residue was added the solution of the first extract. The resulting solution was mixed with one-fifth of its volume of water. The mixture was agitated in the Vortex mixer. After centrifugation two phases separated. The lower phase was shaken with two-fifths of its volume of $CHCl_3$–CH_3OH–0.74% aqueous KCl (3:48:47, v/v), and two phases were separated again by centrifugation (Folch *et al.*, 1957).

The two upper aqueous phases were combined. The combined aqueous phase contains most of the higher gangliosides with the exception of G_{M3}, which is about equally distributed between the aqueous phase and the lower organic phase. (See Mora *et al.*, 1969, and also below.) The aqueous phase was dialyzed against distilled water at about 4°. The retentate was taken to dryness by lyophilization, and the residue was dissolved in about 10–15 ml of warm CH_3OH. The solution was gradually concentrated (40°, N_2) in a conical tube to *ca.* 100 μl, and the whole solution was applied from a 50-μl syringe in a 2-cm long line onto a thin-layer chromatography plate. To complete the transfer the tube and the syringe was rinsed with 50 μl of CH_3OH.

The lower (organic) phase was brought similarly to dryness (40°, N_2), and the residue was taken up on 2 ml of 0.2 M NaOH–CH_3OH and saponified at room temperature for 1 hr. The solution was neutralized with 1 M aqueous acetic acid, and $CHCl_3$–CH_3OH (2:1, v/v) (15–20 ml) was added to produce a clear solution. Water, one-fifth volume, was added, and the mixture was thoroughly agitated, and the two phases were separated by centrifugation. The lower phase was mixed with two-fifth volume of $CHCl_3$–CH_3OH–H_2O (3:48:47, v/v), the mixture was agitated and the two phases were separated again. The lower phase was similarly concentrated to *ca.* 100 μl and applied to the same thin-layer plate as the upper phase, as described above.

The chromatography plate (Analtech, 20 × 20 cm) was a precoated silica gel G (250 or 1000 μ) depending on the total protein content of the extracted cells. Known amounts of ganglioside standards (G_{D1a}, and G_{M1} dissolved in water; G_{M3} dissolved in $CHCl_3$–CH_3OH, 2:1, v/v) were applied to the same plate. Ganglioside standards were obtained as follows. G_{D1a} and G_{M1} were isolated from beef brain, G_{M2} from human brain tissue from patients with Tay-Sachs disease (Kolodny *et al.*, 1970), and G_{M3} from dog erythrocytes (Cumar *et al.*, 1970). Plates were developed (~3 hr) with freshly prepared $CHCl_3$–CH_3OH–2.5 M NH_4OH (60:35:8, v/v).

After development and drying, the gangliosides were

visualized on the plate by spraying with 0.005% aqueous Rhodamine 6GO (Chroma-Ges. Stuttgart (Stahl, 1969; Weinstein *et al.*, 1970)). The gangliosides appeared as light spots on a pink background, and the areas were marked out on the *wet* plates under uv light. After the plates were dried the spots were scraped off and the gel was transferred to small filter funnels (0.5 cm $\phi \sim$ 3.5 cm long $\sim$ 0.2-cm ϕ tip) manufactured from disposable Pasteur pipets and plugged with glass wool. Rhodamine was eluted with $CHCl_3$–CH_3OH (4:1, v/v) until the gel was colorless. The gangliosides were washed out with $CHCl_3$–CH_3OH (1:1, v/v) containing 5% water (Weinstein *et al.*, 1970), using five 400-μl portions and a slight air pressure to accelerate the eluting process. The ganglioside solutions were collected and were brought to dryness (40°, N_2) in 2.5-ml tubes.

Methanolysis, N-Acetylation, and Trimethylsilylation of Gangliosides. The residues were dissolved in 2 ml of freshly prepared dry ~0.5 M HCl–CH_3OH and the gangliosides were subjected to methanolysis at 80° for 24 hr (Sweeley and Walker, 1964; Clamp *et al.*, 1967). To ensure anhydrous conditions and to prevent loss by evaporation, the small tubes containing the ganglioside solutions were placed inside a 100-ml bottle which contained about 10 ml of dry ~0.5 M HCl–CH_3OH and the closed bottle was placed in an 80° water bath. After methanolysis the solutions were cooled and concentrated (40°, N_2) to 0.5 ml. The fatty acid methyl esters, liberated during the methanolysis, were extracted three times with 0.5 ml of *n*-hexane. During methanolysis deacetylation of the amino sugars occurs. To reacetylate, the methanolic phases were brought to dryness (40°, N_2) and the residues were taken up in 0.5 ml of freshly prepared acetic anhydride–CH_3OH (1:3, v/v) with an added pinch of CH_3COOAg (Sweeley and Walker, 1964; White, 1940). After 24 hr at room temperature, in a similar closed system as above, the re-N-acetylation of galactosamine and neuraminic acid is complete (Clamp *et al.*, 1967). Silver acetate was removed by filtration using the small improvised filter funnels and the silver acetate was thoroughly washed with dry CH_3OH using at least six 100-μl portions. One by one the 100-μl aliquots were brought to dryness (40°, N_2) in a small Kimax tube (0.5 × 5 cm). Traces of acetic anhydride were removed with solid KOH overnight in a vacuum desiccator

To obtain trimethylsilyl derivatives the dry residues were dissolved in freshly prepared 50 μl of dry pyridine–hexamethyldisilazane–trimethylchlorosilane (5:1:1, v/v). After 1–2 hr at room temperature 1- to 3-μl aliquots were injected with a 5-μl syringe into the gas chromatograph (see below). (It is important to determine and to deduct the remaining variable liquid volumes in the needle of the syringe by back-suctioning it into the emptied syringe.)

Gas Chromatography. An F&M Model 810 gas chromatograph, equipped with hydrogen flame detectors and dual, coiled glass columns (5 ft; internal diameter 0.25 in), was used. Columns were packed with 2.5% silicone rubber SE-30 on Chromosorb W, 100–120 mesh (Hewlett Packard, Avondale Division, Palo Alto, Calif.). Packing was performed by applying vacuum and vigorous vibration with the Vortex mixer. Columns were conditioned initially at 250° without gas flow (*ca.* 10 hr, disconnected from the detector) and then at 220° with normal gas flow (see below) up to several days. After conditioning breaks up to 1 cm length appeared in the packing material. The columns were removed from the instrument, and the application of vacuum and vigorous vibrating procedure was repeated until these breaks disappeared. To bring the shortened packing material to its original length a small amount of fresh packing material was added at the injection side. Final conditioning of the column was then carried out by injecting the silylating reagent and employing the temperature program and gas flow given below.

TABLE I: Retention Times[a] and Elution Temperatures of Carbohydrates by Gas–Liquid Chromatography.

Methyl D-galactofuranoside	0.90	153
Methyl α-D-galactopyranoside	0.93	155
Methyl β-D-galactopyranoside	0.97	157
Methyl α-D-glucopyranoside	1.00[a]	159
Methyl β-D-glucoypranoside	1.04	161
N-Acetylmethyl-D-galactosaminide A	1.19	170
N-Acetylmethyl-D-galactosaminide B	1.25	174
Sialic acid–methyl glycoside methyl ester A	1.71	200
Sialic acid–methyl glycoside methyl ester B	1.76	204

[a] Retention times, relative to α-methyl glucopyranoside. Analytical conditions and instrumental settings are given in the text; temperature program, 100–210°.

The following gas chromatography conditions were found appropriate for suitable separation and quantitation of the carbohydrate derivatives from the small amounts of gangliosides present: temperature program, 100–210°, 1°/min; injection port temperature, 22°; detector temperature, *ca.* 320°; range, 10; attenuation, 2; chart paper speed, 1 in./4 min; nitrogen (high purity), 60 psi (*ca.* 40 ml/min); air, 24 psi (*ca.* 360 ml/min); hydrogen (high purity), 10 psi (*ca.* 30 ml/min). It was found advantageous to maintain the columns overnight at 100° and to flush the columns with the gas mixture each morning by one or two blank runs (2°/min) before starting the ganglioside determinations.

Quantitation of Carbohydrates. To allow identification and quantitation of the carbohydrate peaks from the gas chromatogram, authentic carbohydrate derivatives were synthesized. *O*-Methyl glycosides were synthesized from D-glucose (J. T. Baker Chemical Co., Phillipsburg, N. J.), D-galactose (Nutritional Biochemicals Corp., Cleveland, Ohio), *N*-acetyl-D-galactosamine (Sigma Chemical Co., St. Louis, Mo.), and from sialic acid (Pierce Chemical Co., Rockford, Ill.). Since during the treatment with anhydrous CH_3OH and HCl, the acetyl groups are cleaved off from the amino sugars (see above), the methylgalactosaminide and the deacetylated methyl glycoside methyl ester of sialic acid were subjected to the identical acetylation conditions given above for the gangliosides. The re-N-acetylation was found necessary for gas chromatography since the *O*-methyl glycosides of silylated galactoside and galactosaminide could not be separated by the temperature program, similarly to the isothermal experiments reported before (Sweeley and Walker, 1964).

Measured aliquots of the carbohydrate derivatives were subjected to gas chromatography under the temperature program specified above. Multiple sharp peaks were obtained. The number of the peaks and their sequence was generally similar to that reported before, under different conditions (Sweeley and Walker, 1964; Clamp *et al.*, 1967). The peak areas were assigned to the appropriate anomers using authentic methyl α-D-glucopyranoside and methyl α-D-galactopyranoside standards. The retention times and the elution temperatures of the various carbohydrate derivatives is given in Table I.

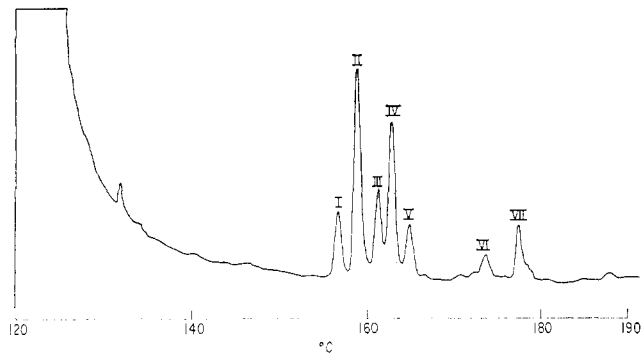

FIGURE 1: Gas chromatogram of G_{M1} ganglioside. An authentic sample of G_{M1} ganglioside (50 μg) was first subjected to thin-layer chromatography and recovered as given in the Experimental Section. Then it was subjected to methanolysis, re-N-acetylation, and silylation in 26 μl of silylation mixture, also as described. This mixture (2 μl) was then injected into the gas chromatograph and programmed as described under Materials and Methods, except the temperature range was 120–190°. This resulted in slightly higher elution temperatures of the carbohydrate derivatives, as compared to the 100–210° temperature program (*cf.* Table I and Figure 3). Peak I, methyl D-galactofuranoside; II, methyl α-D-galactopyranoside; III, methyl β-D-galactopyranoside; IV, methyl α-D-glucopyranoside; V, methyl β-D-glucopyranoside; VI, *N*-acetylmethyl-D-galactosaminide A; VII, *N*-acetylmethyl-D-galactosaminide B. In this experiment the sialic acid peaks did not appear since the end temperature was below their elution temperature.

Method for Identification and Quantitation of Gangliosides. Known amounts of an authentic G_{M1} and of an authentic sialic acid sample were subjected to a standardized carbohydrate derivation procedure. The condition of methanolysis, re-N-acetylation, and trimethylsilylation was the same as given above for the gangliosides obtained from the cell materials. A measured aliquot of the resulting silylated products from G_{M1} gave a gas chromatogram reproduced in Figure 1. Chromatograms were also obtained from authentic silylated sialic acid, programmed for higher temperatures. Two sharp sialic acid peaks appeared with the elution temperature specified in Table I. The peaks in Figure 1 were assigned to the respective carbohydrate anomers on the basis of separate gas chromatography experiments with the authentic carbohydrate derivatives as reported above. The number of peaks for each carbohydrate representing the anomers were: three for galactose, two for glucose, two for acetylgalactosamine (Table I). Peak areas were determined by triangulation. Total amount of each carbohydrate present was proportional to the combined areas of the peaks representing the anomers. The ratio of carbohydrates in G_{M1} was near to the theoretical value of 1:2:1 glucose:galactose:*N*-acetylgalactosamine.

To estimate the amount of recoverable gangliosides on the basis of the various carbohydrates present as determined by the above procedure, a calibration graph was prepared as described in Figure 2, by gas chromatography of known amounts of authentic G_{M1} and sialic acid samples. The calibration graph was linear for each carbohydrate shown, within the range employed for identification and quantitation of the recovered gangliosides from the various cell lines (see below). Since in certain gas chromatography experiments an unidentified peak appeared which overlapped the *N*-acetylgalactosaminide peaks, galactosaminide peaks were not used for quantitation.

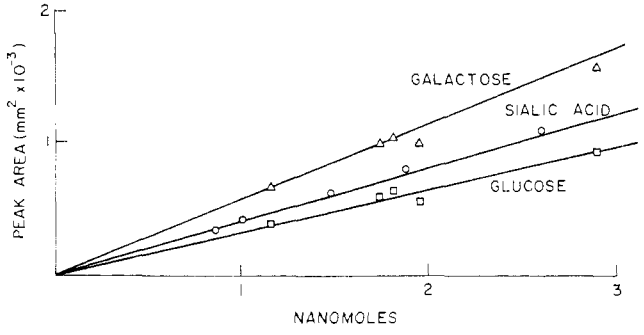

FIGURE 2: Calibration graph for gas chromatography. Authentic sample of G_{M1} and sialic acid was treated with anhydrous HCl–CH_3OH, re-N-acetylated, and silylated as described under Experimental Section, similar to that for cell gangliosides. The measured amounts of aliquots were injected and chromatogrammed with a temperature program of 120–190°, 1°/min, for G_{M1}; and 100–220°, 1°/min, for sialic acid. Peak areas were determined by triangulation, and the identified peaks of the anomeric glycosides (*cf.* Figure 1) were added together and plotted against the applied quantities. The abscissa is given in nanomoles of glucose and sialic acid present. For galactose the nanomoles should be multiplied by two, since there are two glactosyl residues in G_{M1}.

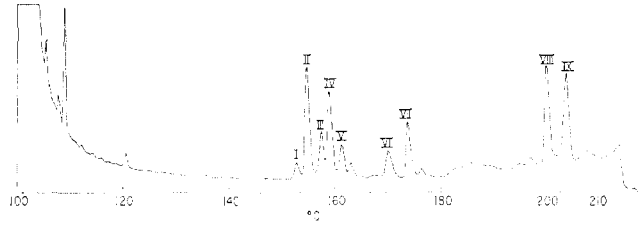

FIGURE 3: Gas chromatogram of a ganglioside (G_{D1a}) from T AL/N cells. The cells were extracted as described under Experimental Section, and subjected to the same analytical conditions as described there and also in the legend for Figure 1, with the exception that the temperature program covered the range of 100–210°. Peaks I–VII are identified in the legend of Figure 1. Peak VIII, sialic acid–methyl ester methyl glycoside A; IX, sialic acid methyl ester methyl glycoside B. Peaks VIII and IX were identified from calibration experiments with authentic sialic acid as in Figure 2.

Results

Determination of Gangliosides in Cell Extracts. Glycolipid extracts were prepared and were subjected to thin-layer chromatography as described above. In each thin-layer chromatography plate known aliquots of authentic gangliosides G_{M3}, G_{M1}, and G_{D1a} were also included. After detection of the ganglioside spots and separate collection and elution, methanolysis re-N-acetylation and trimethylsilylation was carried out as for the authentic samples. Figure 3 gives a representative gas chromatogram obtained from a Rhodamine-staining spot which in the thin-layer chromatography appeared in the G_{D1a} region from the aqueous phase of a glycolipid extract of T AL/N cells. The peak areas were determined by triangulation, and after summation of the appropriate carbohydrate peaks the ganglioside was identified as G_{D1a} from the ratio 1:2:1:2 of glucose:galactose:*N*-acetylgalactosamine:sialic acid. Then the amount of the G_{D1a} recovered from this area of thin-layer chromatogram of the aqueous phase glycolipid extract was estimated by the calibration graph. Similar experiments were carried out on each Rhodamine-staining spot of the thin-layer chromato-

grams. The carbohydrate ratios generally allowed unambigous identification of the gangliosides.

Table II gives the total amount of the various detectable gangliosides present in the cell lines, as determined by gas–liquid chromatography. In the cell lines T AL/N and SVS AL/N, values based both on the sialic acid peaks and also on the glucose peak are given for comparative purpose. In the Swiss and Balb cell lines the sialic acid peaks were best resolved, therefore these were used for quantitation of the gangliosides.

The amount of gangliosides vary depending on the origin of the cell line. The T AL/N and Swiss 3T3 cells have higher amount of total gangliosides than the Balb 3T3, a trend observed previously under somewhat different cell growth conditions (Brady and Mora, 1970).

The parent cell lines T AL/N (both on the basis of sialic acid and of glucose) and Swiss 3T3 have considerable higher amounts, about ten times more of G_{D1a} than the virally transformed derivative cell lines SVS AL/N and SV 3T3 101, respectively. This ratio is in complete agreement with our previous observation on these (Brady and Mora, 1970) and other cell lines (Mora *et al.*, 1969) as confirmed by others (Sheinin *et al.*, 1971) based on ganglioside estimation by colorimetric techniques on thin-layer chromatograms. The absolute values of gangliosides were slightly different by the two methods. The G_{D1a} values (average) by colorimetric method were: TAL/N = 1.2, SVS AL/N = 0.16; Swiss 3T3 = 1.8, SV 3T3 101 = 0.05 nmole/mg of protein.

Values in Table II on Balb 3T3 cell line show a similar but more moderate, about sevenfold, decrease in the low amount of G_{D1a} present upon virus transformation, similar to that observed previously by thin-layer chromatography in this pair of cell lines after growth in Eagle's medium (G_{D1a} of Balb 3T3 = 0.8, of SV T2 = 0.1 nmole/mg of protein, Brady and Mora, 1970). In the earlier investigation when using the Dulbecco–Vogt medium which was the common medium for all cells in the current set of investigation, the gangliosides were found to be too low for quantitation in the Balb cells by the colorimetric method (Brady and Mora, 1970).

Table II also shows by the more accurate gas chromatography method that in addition to G_{D1a}, both G_{M1} and G_{M2} gangliosides are also lower in amount in the SVS AL/N line, as compared to the parent T AL/N line; also the sum of G_{M1} and G_{M2} is lower in the SV 3T3 101 transformed line as compared to the parent Swiss 3T3 line. These observations are in complete agreement with our finding, that after DNA virus-induced transformation of various cell lines, including the AL/N and the Swiss 3T3 cells, an enzyme which is necessary for the synthesis from G_{M3} (hematoside) of G_{M2} and in turn of the next higher ganglioside homologs G_{M1} and G_{D1a}, the uridine diphosphate *N*-acetylgalactosamine:hematoside *N*-acetylgalactosaminyltransferase is much reduced in activity (Cumar *et al.*, 1970).

In the Balb 3T3 cell line after SV 40 virus transformation the major change seems to be in the decrease of the G_{M1} content as found by gas chromatography. The above-mentioned decrease in G_{D1a} content, however, is discernible, in spite of the low absolute values.

TABLE II: Gangliosides in Normal, Spontaneously Transformed, and Virus-Transformed Mouse Cells.

Cell Type	Values Based on[a]	Gangliosides[c] (nmoles/mg of Protein) G_{D1a}	G_{M1}	G_{M2}	G_{M3}
T AL/N	Sialic acid	0.81	0.52	0.53	1.31
	Glucose	1.00	0.61	0.75	1.69
SVS AL/N	Sialic acid	0.05	0.18	0.28	0.85
	Glucose	0.12	0.21	0.17	0.60
Swiss 3T3	Sialic acid	1.18	1.38[b]		2.38
SV 3T3 101	Sialic acid	0.18	0.85[b]		0.43
Balb 3T3	Sialic acid	0.21	0.14	0.33	0.32
SV T2	Sialic acid	0.04	0.18	0.30	0.65

[a] In Swiss and Balb cell lines the sialic acid peaks were better resolved than the glucose or galactose peaks, therefore only the sialic acid peaks were employed to calculate the ganglioside content. [b] Assignment is unclear, due to poor separation on the thin-layer chromatogram and to insufficiently distinct ratio of the carbohydrates after gas chromatography. [c] Reproducibility was ensured by an internal control, by carrying out quantitation of a known amount of input authentic G_{M1} included in each thin-layer chromatogram. The G_{M1} spot was subjected to the same recovery elution methanolysis extraction re-N-acetylation trisilylation and gas chromatography technique as the cell ganglioside spots. In over 20 independent experiments including different cell lines (and certain cells in triplicate) no significant deviations were observed when estimates from the calibration curve (Figure 2) were compared with the input G_{M1}. Losses starting from thin-layer chromatography step to the end of gas chromatography were 16–21%, as determined by subjecting a known amount of D-glucose to treatment at 80° for 24 hr in 0.5 N HCl–CH_3OH followed by trimethylsilylation and gas chromatography. The losses from the cell homogenization step up to the thin-layer chromatography were found to be about 16% (Mora *et al.*, 1969). These add up to an estimated average loss of ~35% for the various gangliosides in these determinations.

Discussion

The determination of gangliosides by an accurate and chemically direct method in control and tumorigenic DNA virus-transformed mouse cell lines was mandatory for several reasons. First of all, it was necessary to verify the chemical composition of the mouse gangliosides. Secondly, it was essential to show that the colorimetric estimation of gangliosides on the thin-layer chromatograms was indeed valid. The results of the analyses by the gas–liquid chromatographic procedure employed in the present experiments definitely prove that the previously reported drastic decrease in the ganglioside G_{D1a} after SV40 virus transformation of mouse cell lines (Mora *et al.*, 1969; Brady and Mora, 1970) indeed occurs. The ratio of decrease in G_{D1a} content based on gas–liquid chromatography is nearly the same as the ratio of decrease previously estimated by the colorimetric methods.

A temperature elution program for gas chromatography has been found optimal. We have observed that under the recommended (Sweeley and Walker, 1964) isothermal conditions (160°) the *O*-methyl glycoside methyl ester of the re-N-acetylated and silylated sialic acid did not elute from the columns. Also, we have found that the *N*-acetylgalactosaminide had a retention time higher than that reported (Sweeley and Walker, 1964). Also, we have found that the

separation of the galactoside and the glucoside peaks was poor at the recommended isothermal conditions.

The following observations are presented to emphasize experimental details which have to be attended carefully for sensitive and reproducible determinations by the temperature-programmed gas chromatography method. We have found it difficult to achieve complete silylation of the carbohydrates with less than 50 μl of solvent mixture. Because of this relatively large amount of solvent present, we found it advisable to start the program at a low temperature (100°) to elute completely the silylation mixture so that the peaks of the neutral hexoses will be well separated from the solvent tail (*cf.* Figure 3). When the efficiency of the columns started to decrease after prolonged use (several weeks), the galactoside peaks separated poorly, whereas the galactosaminide and sialic acid peaks remained well separated. When this was observed new columns were prepared and conditioned. Care had to be exercised to obtain good compensation for substrate bleed (base-line shift), which often was difficult with the high-sensitivity setting of the recorder and with the high end temperature.

The derivatization of the carbohydrates and of the gas chromatography technique presented above offers an accurate and internally consistent method for the quantitative comparison of the relatively small amount of gangliosides obtainable from tissue-cultured cells. Further studies are in progress on the role of gangliosides in the control of cell growth in tissue culture and *in vivo*.

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References

Brady, R. O., and Mora, P. T. (1970), *Biochim. Biophys. Acta 218*, 308.

Clamp, J. R., Dawson, G., and Hough, L. (1967), *Biochim. Biophys. Acta 148*, 342.

Cumar, F. A., Brady, R. O., Kolodny, E. H., McFarland, V. W., and Mora, P. T. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 757.

Folch, J., Lees, M., and Stanley, G. H. S. (1957), *J. Biol. Chem. 226*, 497.

Hakomori, S. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1741.

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

Klenk, H., and Choppin, D. W. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 57.

Kolodny, E. H., Brady, R. O., Quirk, J. M., and Kanfer, J. N. (1970), *J. Lipid Res. 11*, 144.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. 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.

Sheinin, R., Onodera, K., Yogeeswaran, G., and Murray, R. K. (1971), The Biology of Oncogenic Viruses, IInd Le Petit Symposium, Milan Le Petit, p 274.

Smith, R. W., Morganroth, J., and Mora, P. T. (1970), *Nature (London) 227*, 141.

Stahl, E. (1969), Thin-Layer Chromatography, 2nd ed, New York, N. Y., Springer-Verlag, p 896.

Svennerholm, L. (1963), *J. Neurochem. 10*, 613.

Sweeley, G. C., and Walker, B. (1964), *Anal. Chem. 36*, 1461.

Weinstein, D. B., Marsh, J. B., Glick, M. C., and Warren, L. (1970), *J. Biol. Chem. 245*, 3928.

White, T. (1940), *J. Chem. Soc.*, 428.

Yogeeswaran, G., Wherrett, J. R., Chatterjee, S., and Murray, R. K. (1970), *J. Biol. Chem. 245*, 6718.