Studies on the Glycosphingolipids of Normal and Virally Transformed 3T3 Mouse Fibroblasts*

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SUMMARY

The neutral glycosphingolipids and the gangliosides of normal 3T3 mouse fibroblasts, one polyoma-transformed, and three SV40-transformed clonal isolates have been examined. The normal 3T3 cells contained mono- and dihexosylceramides and a complex pattern of gangliosides. The carbohydrate and fatty acid compositions of these neutral glycosphingolipids and of the principal gangliosides were determined and partial structures for these compounds are proposed. One polyoma-transformed (3T3-Py6) and one SV40-transformed derivative cell line (3T3-SV-479) were found to exhibit marked simplification of ganglioside pattern as compared with the control cells, a finding in agreement with that of a previous study (BRADY, R. O., AND MORA, F. T. (1970) Biochem. Biophys. Acta 218, 308). Two other clonal isolates of SV40-transformed cells (3T3-SV-A26 and 3T3-SV-CE56) exhibited complex patterns of gangliosides, generally similar to those found in the control cells, but showing a marked increase of the ganglioside GD3. Possible explanations for these observations are discussed. No correlation was detected between the complexity of the glycosphingolipid pattern and the saturation density obtained with the cells studied. Experiments using [1-14C]-glucosamine as a precursor confirmed the ability of control and transformed cells to synthesize their complement of gangliosides. Plasma membrane fractions of 3T3 cells were found to be enriched 3-fold with respect to the various gangliosides detected in the whole cells. Certain gangliosides of the control and 3T3-Py6 cells were susceptible to the action of neuraminidase in a membrane-bound state.

A considerable body of evidence indicates that alterations of the cell surface occur during the phenomenon of malignant transformation (cf. 1, 2). Various components of the cell surface have been examined in recent years in attempts to define the chemical nature of these changes. Of specific interest in relation to the present work have been the glycosphingolipids. The studies of Rapport et al. (3, 4) first demonstrated alterations in the pattern of neutral GSLs in tumor as compared with normal tissues. These observations have now been extended to the gangliosides of cells cultivated in vitro; hamster cells (BHK-21) transformed by polyoma virus (7), AL/N mouse epithelial cells transformed by SV40 virus (8), 3T3 mouse fibroblasts transformed by polyoma and SV40 viruses (9), and chick embryo fibroblasts transformed by Rous sarcoma virus (10). In these studies it was found that the ganglioside pattern of the virus-transformed cells was generally simpler than that of the control cells. A similar simplification of ganglioside pattern was observed in chemically induced hepatoma cells as compared with normal hepatocytes, both grown in vitro (11) and in vivo (12, 13). Deficiencies in the activity of specific glycolipid glycosyltransferases have been demonstrated (14-17) that could account for the alterations of GSL patterns observed in virus-transformed cells.

Studies using radioactive precursors have shown that in cultivated hamster cells the rate of synthesis of certain GSLs differs in growing cells as compared with nongrowing, densely packed cells (18-21). In the case of virus-transformed cells, the stage of the cell growth cycle had no apparent effect on the rate of synthesis of these GSLs.

Some information has been obtained concerning the cellular localization, chemical composition, and structure of GSLs in cultured mammalian cells. Neutral GSLs have been shown to be enriched in plasma membrane fractions (22). The speculation that the gangliosides might also be located mainly in surface membranes (7, 11) has received support from the observations that plasma membrane preparations of various cultured animal cells are significantly enriched in these compounds (22-24) and also from immunological studies (25). Partial characterization of the gangliosides of monkey kidney cells (22), mouse L-cells (23, 26), mouse embryo secondary cultures (26), various mouse fibroblastic lines (27), and mouse neuroblastoma cells (28) have been undertaken. The main GSL of hamster (BHK-21) kidney fibroblasts has been identified as N-acetylneuraminylactosylceramide (7). The work presented here is an extension of

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† The abbreviations used are: GSL, glycosphingolipid; LBSA, lipid-bound sialic acid; PBS-EDTA, isotonic phosphate-buffered saline (0.9%) (5) containing 0.01 M sodium EDTA; N-AN, N-acetyl neuraminic acid; N-GS, N-glycolyl neuraminic acid. The nomenclature used to describe the gangliosides is that of Svennerholm (6).
Chemical Co., Chicago, Illinois) at a concentration of about
medium (Connaught Medical Research Laboratories, Toronto,
inoculated into 32-ounce Brockway bottles in 60 ml of 1066
surface, combined in PBS, and collected by centrifugation. The
as described below.
three times with PBS. The cells were scraped from the growth
medium was decanted and the cell layers were washed
ounce Brockway bottles and were harvested as follows. The
growth medium was decanted and the cell layers were washed three
times with 20.ml of PBS and were treated with 0.1% trypsin solution
by microscopic examination. They were further incubated for
48 hours with no medium change. The medium was then
decanted, and the cell layers were washed three times with 20.ml
bottles. They were grown at 37° in an incubator flushed with 95% air + 5% CO2 to confluence, as judged
fluence and then detached from the growth surface with PBS-
fluenced cell sheets. No such growth was detected in those plates
to which were added the normal 3T3 cells.

Properties of Cells

Saturation Density—To measure saturation density, cells were
incubated into 32-ounce Brockway bottles in 60 ml of 1066
medium (Connaught Medical Research Laboratories, Toronto,
Ontario) supplemented with 7.5% fetal calf serum (Reheis
Chemical Co., Chicago, Illinois) at a concentration of about
1.2 x 10^5 cells per bottle. They were grown at 37° in an incubator flushed with 95% air + 5% CO2 to confluence, as judged
by microscopic examination. They were further incubated for
48 hours with no medium change. The medium was then
decanted, and the cell layers were washed three times with 20.ml
volumes of PBS and were treated with 0.1% trypsin solution
(32) to release single cells which were then counted.

T-Antigen—The production of nuclear SV40-T antigen was
assessed by the technique described by Pope and Rowe (34),
using anti-T serum obtained from Dr. Vittorio Defendi (the
Wistar Institute).

Tumor Formation—The tumorigenicity of the various 3T3
cells under study was also examined. Groups of 10 young male
Swiss mice were inoculated subcutaneously with 5 x 10^5 to
5 x 10^6 cells. The animals were examined periodically during
the following 12 months for the presence of tumor nodules and
were killed when nodules were easily palpable.

Growth of 3T3 Cell Derivatives on Irradiated 3T3 Cells—The
capacity of the various 3T3 cells to grow on irradiated 3T3 cell
layers was examined essentially by the procedure of Schutz and
Mora (35). 3T3 cells (5 x 10^5 cells per 65-mm Falcon Petri dish) were irradiated (20,000 rads) 24 hours after plating. They
were then used as feeders upon which were plated between 200
and 1,000 cells in triplicate plates. The cultures were incubated
for 10 days, after which they were stained with methylene blue.
The extensive growth of all of the virus-transformed cells on
the 3T3 feeder cells was apparent in the deeply staining con-
fluent cell sheets. No such growth was detected in those plates
to which were added the normal 3T3 cells.

Support of BEV-1 Virus—It has been demonstrated by Taylor
et al. (36) that tumor cells and virus-transformed cells are
susceptible to the bovine enterovirus BEV-1, whereas normal
cells are relatively resistant. This virus (obtained from Dr. C. P.
Stanners, Ontario Cancer Institute) was used to further examine
the phenotypic properties of the transformed cells under study.
The capacity of this virus to grow in these cells was assessed by
plaque assay (cf. 37) or by its cytopathic effect. The latter was
measured as follows. Cells were plated at 2 x 10^5 cells per
65-mm Petri dish. After 48 hours the cells were just confluent.
They were infected with 0.1 ml of BEV-1 stock containing
3 x 10^5 plaque-forming units per ml as titered on mouse L-cells.
The cultures were examined daily for cellular degeneration.

Agglutination by Concanavalin A—The procedure of Inbar
and Sachs (38) was used to assess the sensitivity of the various
3T3 cell types to concanavalin A. Cells were grown to con-
fluence and then detached from the growth surface with PBS-
EDTA solution. The resulting suspension consisted of a major-
ity of free cells with occasional clumps. The cell suspensions, in
nontissue culture Petri dishes, were incubated with 10, 100, 500,
and 1000 μg per ml of concanavalin A (Calbiochem, Los Angeles,

Materials and Methods

Cells

The cell lines under study (see Table I) were derived from a
clone of 3T3 mouse fibroblasts (29) picked by Dr. Marguerite
Vogt (the Salk Institute). They are designated as follows: (a)
normal 3T3 cells, 3T3Py; (b) SV40-transformed 3T3 cells, 3T3-
SV-479 (30), 3T3-SV-A20 (31), 3T3-SV-A26a (31); (c) polyoma-
transformed 3T3 cells, 3T3-PySa (31). The cells were main-
dicated in a cell sheet on the growth surface. In the case of 3T3-
Py6, cells tended to shed from the surface once confluence had
been attained. Numbers in parentheses refer to number of deter-
minations.

Numbers in parentheses indicate number of animals develop-
ing tumors; 10 mice were inoculated for each type of cell studied.

Previous studies (2, 26) and is directed towards examining the
general significance of altered GSL metabolism in virus-trans-
formed cells. Several clonal isolates of such transformed cells
have been examined in studies of GSL composition, metabolism,
and subcellular location.

Table I

Properties of various cell derivatives

<table>
<thead>
<tr>
<th>Cell</th>
<th>Saturation densitya (cells/cm² x 10^2)</th>
<th>T Antigen</th>
<th>Tumor formationb</th>
<th>Growth on irradiated 3T3 cells</th>
<th>Support of BEV-1 growthc</th>
<th>Agglutination by concanavalin A^n</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3</td>
<td>1.13 (2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>3T3-Py6</td>
<td>1.89 (±0.41) (6)</td>
<td>+</td>
<td>(0)</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>3T3-SV-479</td>
<td>5.17 (±0.04) (3)</td>
<td>+</td>
<td>(4)</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>3T3-SV-A20</td>
<td>2.99 (±0.07) (4)</td>
<td>+</td>
<td>(0)</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>3T3-SV-CE66</td>
<td>1.91 (±0.39) (3)</td>
<td>+</td>
<td>(7)</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

a Saturation density was measured only in terms of cells asso-
associated in a cell sheet on the growth surface. In the case of 3T3-
Py6, cells tended to shed from the surface once confluence had
been attained. Numbers in parentheses refer to number of deter-
minations.

b Numbers in parentheses indicate number of animals develop-
ing tumors; 10 mice were inoculated for each type of cell studied.

Numbers in parentheses refer to number of animals develop-
ing tumors; 10 mice were inoculated for each type of cell studied.

c The ability to support BEV-1 growth, as determined by either
plaque assay or observation of cytopathic effects, is scored in an
all or none manner.

d ±, no detectable agglutination under the conditions employed
until >500 μg per ml of lectin was employed; +++, detectable ag-
glutination within 30 min at room temperature using 10 μg per ml
de of lectin.

§ The production of nuclear SV40-T antigen was
assessed by the technique described by Pope and Rowe (34),
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and 1000 μg per ml of concanavalin A (Calbiochem, Los Angeles,
Cells were grown just to confluence and were then incubated in 20 ml of medium containing 0.1 μCi per ml of n-[1-14C]glucosamine-hydrochloride (New England Nuclear, 51.4 mCi per μmol). After 24 hours they were harvested using PBS containing 0.1 PCi per ml of n-[1-14C]glucosamine, as Swiss mice (from which the 3T3 cells used for the tumorigenicity study) are not highly inbred animals. This observation does not necessarily mean that these cells were nontumorigenic, as Swiss mice (from which the 3T3 cells used for the tumorigenicity study) are not highly inbred animals.

**Plasma Membrane Isolation**

The procedure used to isolate a plasma membrane fraction (adapted from that of Brunette and Till (39)) has been described previously (33). Briefly, cells grown to confluence were dissociated to form a suspension of single viable cells and were treated with hypotonic zinc chloride solution to harden the plasma membrane. The latter was stripped from the congealed cytoplasm and nuclei by homogenization and was purified by protein assays were performed on aliquots of the cell pellets, and nonlipid residue fractions by the method of Lowry et al. (40); bovine serum albumin was used as a standard.

**Solvents**

All solvents were of analytical grade. Chloroform and methanol were redistilled prior to use.

**Isolation of Ganglioside and Neutral GSL Fractions**

Gangliosides were extracted from the cell pellets and membrane fractions by the procedure of Suzuki (41). LBSA in the ganglioside fraction was estimated by the procedure of Svennerholm (12) as modified by Miethke and Talalay (43). The neutral GSLs present in the lower phase of the Folch extracts obtained during the procedure for ganglioside isolation were separated by a modification of the procedure of Vance and Sweeley (44) as described previously (26).

Gangliosides were separated and detected by thin layer chromatography on glass plates coated with Silica Gel G as described previously (45); the standard solvent system was chloroform-methanol-ammonia-water (60:35:1, by volume). The sialic acid content of individual ganglioside bands was quantitated by the method of MacMillan and Wherrett (46). Neutral GSLs were separated on glass plates coated with Silica Gel G by development in chloroform-methanol-water (65:25:4, by volume) and were visualized with the aniline-diphenylamine reagent (47). For purposes of structural studies, gangliosides and neutral GSLs were separated in these systems, detected by spraying with methanol-water (1:1, by volume), and eluted from the silica gel with chloroform-methanol-water (60:35:8, by volume).

**Partial Acid Hydrolysis of Gangliosides and Identification of the Sialic Acid and GSL Products**

Partial acid hydrolysis of individual gangliosides purified by preparative thin layer chromatography was performed as described previously (26).

**Neuraminidase Treatment of Isolated Gangliosides and Intact Cells**

The effect of neuraminidase treatment on the principal gangliosides of control and transformed cells was studied to assist in the characterization of these compounds. Aliquots (containing 30 to 40 nmoles of LBSA) of the purified gangliosides corresponding in chromatographic migration to GM3 and GD1α, obtained from 3T3 cells, were dried under nitrogen in conical tubes. To the dry residues was added 0.1 ml of sodium acetate (0.1 M, pH 5.3) containing 0.85% NaCl and 0.005 M CaCl2 and 20 i.u. of stock *Vibrio cholerae* neuraminidase (500 i.u. per ml, from Behring Diagnostics Inc., Woodbury, New York). The mixtures were agitated vigorously, covered with 0.1 ml of toluene (to prevent bacterial contamination) and incubated at 37° for 72 hours. The material was then dried and subjected to thin layer chromatography on glass plates coated with Silica Gel G in chloroform-methanol-ammonia-water (55:40:2:10, by volume). The plates were sprayed with resorcinol to detect released sialic acids and residual gangliosides, and resprayed with aniline-diphenylamine to detect the other GSL products. Similar studies were performed on the GM3 species of 3T3-SV-479 and 3T3-Py6 cells.

To determine whether certain of the gangliosides of 3T3 cells were susceptible to neuraminidase in their membrane-bound state, the following experiments were performed. 3T3 and 3T3-Py6 cells approaching confluence (approximately 1 × 10^6 cells per 32-ounce Brockway bottle) were incubated in 20 ml of medium containing 2 μCi of [1-14C]glucosamine for 24 hours. The cells were then harvested by detachment with PBS-EDTA and glass beads. The cell contents of 9 to 10 bottles were pooled. The total cell protein corresponded to approximately 40 mg. The cells were divided into two batches, one serving as a control, the other as the test batch to be treated with neuraminidase.

Each batch of cells was suspended in 5 ml of 0.85% NaCl, 0.005 M CaCl2 (pH 7.0) and 125 i.u. of the stock enzyme preparation
were added to the test batch. The cells were incubated at 37°C for 3 hours with mild shaking. The cells appeared morphologically intact at the end of the incubation period when viewed by phase-contrast microscopy and were impermeable to trypan blue. The cells were pelleted, the supernatant retained for sialic acid analysis, and ganglioside and protein analyses were performed on the cell pellets as described above. Suitable aliquots of gangliosides from control and neuraminidase-treated cells were subjected to thin layer chromatography, and autoradiography of the chromatogram plates was performed prior to staining with resorcinol.

The effect of neuraminidase on the LBSA content of cells in situ was also investigated. 3T3 or 3T3-Py6 cells were grown to confluence and the medium was decanted. The cell layers were washed gently with PBS and 10 ml of 0.85% NaCl-0.005 M CaCl₂ (pH 7.0) was added to each bottle of control and test cells. The stock enzyme solution (40 i.u.) was added to each bottle of test cells, and all cells were incubated at 37°C for 3 hours. The cell layers were intact at the completion of incubation, and the cells appeared unchanged as evaluated by phase-contrast microscopy. Cells were harvested with PBS-E1DTA and glass beads, and the LBSA and protein content of each batch of cells was analyzed.

**Gas-Liquid Chromatographic Analyses of Carbohydrates and Fatty Acids**

The sugar composition of purified individual neutral GSLs and gangliosides was determined by gas-liquid chromatographic analyses of the trimethylsilyl ethers of the O-methyl glycosides as described by Vance and Sweeley (44) and Clamp et al. (48). The amino-sugars and sialic acid species were N-acetylated again with acetic anhydride as described by Clamp et al. (48) prior to trimethylsilylation, but this step was omitted for analysis of the neutral GSL samples (which contained neither amino-sugars nor sialic acids). Analyses were performed on an F and M model 402 gas chromatograph equipped with a glass column (6 feet × ⅛ inch outer diameter) packed with 3.5% SE 30 on 80/100 mesh Diatoport S. The instrument was operated isothermally at 160°C for the neutral GSL analyses and programmed from 160 to 210°C at 1°C per min for the ganglioside analyses. Retention times and correction factors for losses during the entire operation of the various sugars present in the gangliosides were established by analyses of individual sugar standards carried through the same derivatization procedure. Quantitation of the sugar peaks was performed by cutting individual peaks from the chromatogram and weighing them. Analysis of a purified GM₁₂ species from the brain of a patient with Tay-Sachs disease yielded the expected sugar stoichiometry.

The methyl esters of the fatty acids obtained in the hexane phase using the above procedure (44) were analyzed in the same instrument on a glass column (6 feet × ⅛ inch outer diameter) packed with 0% dicetylhexyl glycol succinate on 80/100 mesh Chromosorb W. Separation was performed isothermally at 160°C. Fatty acids were identified by comparison of retention times with NIH standards D and E (obtained from Applied Science Laboratories, Inc., State College, Pennsylvania). For presentation of the percentage composition of the fatty acids, individual peaks were cut out and weighed and the weight of each peak was expressed as a percentage of the total. Quantitative results with NIH standard D agreed with the stated composition data, with a relative error less than 1.3 for major components (>10% of the total mixture) and less than 3.4% for minor components (<10% of the total mixture). It should be noted that separate analysis of hydroxy fatty acids was not performed, although mass spectrometric analysis (vide infra) suggested their presence.

**Mass Spectrometric Analysis of GM₁₂**

The ganglioside corresponding in chromatographic migration of GM₁₂ was purified separately from 3T3 and 3T3-SV-479 cells by a combination of unci column chromatography and preparative thin layer chromatography. The purity of the compound was checked by thin layer chromatography in the standard solvent system for ganglioside analysis and also in propanol-ammonia-water (6:2:1, by volume). In both systems the compound was observed to be free of contaminants and migrated as a double zone. The carbohydrate stoichiometry of this compound is shown in Table V. Mass spectrometric analysis of the trimethylsilylated derivative was kindly performed by Dr. C. C. Sweeley, Michigan State University. The derivatization procedure and operating conditions of the LKB 9000 single-focusing mass spectrometer were as previously described by Dawson and Sweeley (49). Interpretation of the fragmentation pattern was based principally on the observations of these workers.

**Sources of Standard Compounds for Thin Layer Chromatography**

Ganglioside standards were prepared from a human brain obtained at autopsy. Cerebroside was also prepared from this source. Neutral GSL standards were obtained from human kidney. N-AE and N-GN were purchased from Sigma Chemical Co., St. Louis, Missouri.

**RESULTS**

**Ganglioside Content of Normal and Virus-transformed 3T3 Cells**

—The results of quantitative determinations of the LBSA content of the control and transformed 3T3 cells used in this study have been presented in Table II, expressed as nmoles of LBSA per mg protein uncorrected for cell number (cf. 10). The analyses revealed that the LBSA content of the various clonal isolates differed appreciably. Whereas 3T3-Py6 and 3T3-SV-A26 cells showed a moderate diminution of LBSA content (some 17 and 26%, respectively) as compared with the control cells, the LBSA content of 3T3-SV-479 cells was approximately equal to that of the control cells and that of 3T3-SV-CL56 cells was some 50% greater. The data in Table II also indicated that mild trypsinization did not affect the LBSA content of either the control or transformed cells.

**Ganglioside Content of Plasma Membrane Fractions Prepared from 3T3 Cells**—Plasma membrane preparations were obtained from 3T3 cells and analyzed for their LBSA content. The LBSA content of three preparations from 3T3 cells was found to be 12.5 ± 1.4 nmoles per mg of protein. This represented an approximate 3-fold enrichment with respect to the LBSA content of whole cells (cf. data in Table II).

**Ganglioside Patterns of Normal and Virus-transformed 3T3 Cells**—Quantitative and qualitative data on the patterns of gangliosides in normal and virus-transformed 3T3 cells were obtained as shown in Fig. 1 and Table III, respectively. Relatively complex patterns of gangliosides were found in 3T3 cells (Fig. 1A, Slot 3). Prominent resorcinol-positive bands, corresponding in chromatographic migration to GM₁₂, GM₁, and GD₁₄ were evident, while a minor band corresponding in migration to GM₁₃ was also visible. Quantitation of the distribu-
tion of sialic acid in each of these bands (Table III) confirmed the predominance of GM₃, GM₁, and GD₁₉ species. A plasma membrane fraction (Fig. 1A, Slot 3) prepared from 3T3 cells showed a similar pattern to that of the whole cells, while the quantitative analyses revealed relatively minor differences in the distribution of sialic acid among the individual ganglioside bands as compared with the pattern of the whole cells. In contrast, the ganglioside patterns of 3T3-SV-479 cells (and of a plasma membrane fraction prepared from these cells) (Fig. 1A, Slots 4 and 5) were much less complex, consisting principally of compounds corresponding in migration to GM₁ and GM₅. This observation was corroborated by the quantitative analyses on the whole cells presented in Table III. Similarly, the ganglioside pattern of 3T3-Py6 cells (Fig. 1B, Slot 2, and Table III) was also relatively simple, comprised almost entirely of a substance corresponding in migration to GM₁. It was thus of interest to extend these observations to two other clones (A26 and CE56) of SV40-transformed cells (Fig. 1C, Slots 1 and 2, and Table III). These cells were found to exhibit patterns of gangliosides comparable in complexity to that of control cells, but they displayed a marked increase (approximately 3-fold) in the relative amount of the ganglioside corresponding in migration to GD₁₉.

**Studies on Structure of Purified Gangliosides Using Mild Acid and Neuraminidase Hydrolysis**—As a preliminary attack on the structural analysis of the gangliosides of various 3T3 cell derivatives, studies were made of the products derived from hydrolysis by mild acid and by *Vibrio cholerae* neuraminidase. The gangliosides corresponding in chromatographic migration to GM₁ and GD₁₉ obtained from 3T3 cells and that corresponding in migration to GM₅ from 3T3-SV-479 and 3T3-Py6 cells were purified by preparative thin layer chromatography and subjected to mild acid and neuraminidase treatment. As shown in Fig. 2 the GM₅ species from 3T3 cells (Fig. 2A, Channel 1), when subjected to mild acid hydrolysis, yielded compounds correspond-

### Table II

**Lipid-bound sialic acid content of normal and virus-transformed 3T3 cells**

<table>
<thead>
<tr>
<th>Cells analyzed</th>
<th>LBSA*</th>
<th>nmoles/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3 Scraped</td>
<td>4.2 ± 0.3</td>
<td>(4)</td>
</tr>
<tr>
<td>3T3 Trypsinized</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>3T3-Py6 Scraped</td>
<td>3.5 ± 0.2</td>
<td>(4)</td>
</tr>
<tr>
<td>3T3-Py6 Trypsinized</td>
<td>3.2</td>
<td>(1)</td>
</tr>
<tr>
<td>3T3-SV-479 Scraped</td>
<td>4.3 ± 0.4</td>
<td>(4)</td>
</tr>
<tr>
<td>3T3-SV-479 Trypsinized</td>
<td>3.9</td>
<td>(1)</td>
</tr>
<tr>
<td>3T3-SV-A26 Scraped</td>
<td>3.1 ± 0.1</td>
<td>(3)</td>
</tr>
<tr>
<td>3T3-SV-A26 Trypsinized</td>
<td>3.3</td>
<td>(1)</td>
</tr>
<tr>
<td>3T3-SV-CE56 Scraped</td>
<td>6.5 ± 0.2</td>
<td>(4)</td>
</tr>
<tr>
<td>3T3-SV-CE56 Trypsinized</td>
<td>6.7</td>
<td>(1)</td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± standard deviation where sufficient samples were analyzed.

* Cells were harvested by a 5-min treatment with 1% trypsin in 0.44% sodium citrate-0.85% NaCl, pH 7.6.

* Figures in parentheses refer to number of batches of cells used in the analyses.

### Table III

**Distribution of sialic acid among individual ganglioside fractions of normal and virus-transformed 3T3 cells**

Gangliosides were separated by thin layer chromatography and the sialic acid in each fraction was determined by the method of MacMillan and Wherrett (46) using N-AN as a standard. Results are expressed as a percentage of the sialic acid recovered from the plates (approximately 75 to 80%). The data are mean values of duplicate analyses on three batches of cells (unless otherwise indicated by numbers in parentheses) with standard deviations shown where applicable.

<table>
<thead>
<tr>
<th>Material analyzed</th>
<th>GM₁</th>
<th>GM₃</th>
<th>GM₅</th>
<th>GM₁</th>
<th>GD₁₉</th>
<th>GD₁₉ to origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3 Cells</td>
<td>47.5 ± 6.2</td>
<td>7.3 ± 0.2</td>
<td>18.8 ± 1.5</td>
<td>26.4 ± 4.9</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td>3T3 Cell plasma membrane</td>
<td>59.2 ± 1.0</td>
<td>5.3 ± 0.5</td>
<td>15.3 ± 0.3</td>
<td>20.2 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-Py6 Cells (2)</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-SV-479 Cells</td>
<td>83.8 ± 1.0</td>
<td>17.2 ± 1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-SV-A26 Cells (2)</td>
<td>13.2</td>
<td>5.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-SV-CE56 Cells</td>
<td>14.0 ± 0.9</td>
<td>3.9 ± 0.1</td>
<td>7.6 ± 0.5</td>
<td>65.7 ± 1.9</td>
<td>8.8 ± 1.0</td>
<td></td>
</tr>
</tbody>
</table>

* GM₁: Ganglioside of human brain (1), 3T3 cells (2), plasma membrane fraction of 3T3 cells (3), 3T3-SV-479 cells (4), and plasma membrane fraction of 3T3-SV-479 cells (5). GM₃: Gangliosides of human brain (1) and 3T3-Py6 cells (2). GD₁₉: Gangliosides of 3T3-SV-A26 cells (1), 3T3-SV-CE56 cells (2), and human brain (3). All aliquots of gangliosides corresponding in amount to approximately 80 nmoles of LBSA were spotted in each channel. The plates were developed in chloroform-methanol-ammonia-water (60:35:1:7, by volume) and sprayed with resorcinol reagent. The system of nomenclature for the gangliosides is that of Svennerholm (6). The minor band in the position of GD₁₉ seen in the cell extracts (e.g. Channel 2, Fig. 1A) did not yield detectable amounts of sialic acid. The band corresponding in chromatographic migration to GT (e.g. Channels 2 to 5, Fig. 1A) was yellow and is a contaminant derived from dialysis tubing.
FIG. 2. Thin layer chromatograms illustrating the effects of mild acid and neuraminidase hydrolyses on the purified GM₃ species derived from normal and virus-transformed 3T3 cells. A, 3T3 cell GM₃, unhydrolyzed (1); 3T3 cell GM₃, acid-hydrolyzed (2); 3T3 cell GM₃, neuraminidase-hydrolyzed (3); 3T3-Py6 cell GM₃, unhydrolyzed (4); 3T3-Py6 cell GM₃, acid-hydrolyzed (5); 3T3-Py6 cell GM₃, neuraminidase-hydrolyzed (6). B, 3T3-SV-479 cell GM₃, unhydrolyzed (1); 3T3-SV-479 cell GM₃, acid-hydrolyzed (2); standards of neutral GSLs, gangliosides, and sialic acids (3). C, 3T3-SV-479 cell GM₃, unhydrolyzed (1); 3T3-SV-479 cell GM₃, neuraminidase-hydrolyzed (2); same sample as in Channel 6 of A (4); standards of neutral GSLs, gangliosides, and sialic acids (5). Samples containing purified GM₃ species before and after mild acid or neuraminidase hydrolysis were spotted on thin layer plates of Silica Gel G and developed in chloroform-methanol-ammonia-water (55:40:2:10, by volume). The plates were first stained with resorcinol reagent and then with the aniline-diphenylamine reagent. Gangliosides and sialic acids appeared purple and neutral GSLs blue. The aberrant chromatographic migration of N-AN in Channels 5 and 6 of A is due to complexing with Ca²⁺ ions present in the hydrolysis mixture. CM, monoglycosylceramide; CD, diglycosylceramide; CT, triglycosylceramide; CTT, tetraglycosylceramide; XAN, N-acetylenuraminic acid; NGN, N-glycolylenuraminic acid.

TABLE IV

Analyses of the principal gangliosides of control and virus-transformed 3T3 cells, subjected to acid and enzyme hydrolysis

<table>
<thead>
<tr>
<th>Ganglioside studied</th>
<th>Chromatographic migration of hydrolytic products</th>
<th>Proposed structure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sialic acid</td>
<td>GSL</td>
</tr>
<tr>
<td>3T3 Cells, Band 1</td>
<td>GM₃</td>
<td>N-AN, Diglycosylceramide</td>
</tr>
<tr>
<td>Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3 Cells, Band 4</td>
<td>GD₁₅</td>
<td>N-AN, Tetruglycosylceramide</td>
</tr>
<tr>
<td>Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-SV-479 Cells, Band 1</td>
<td>GM₃</td>
<td>N-AN, Diglycosylceramide</td>
</tr>
<tr>
<td>Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-Py6 Cells, Band 1</td>
<td>GM₃</td>
<td>N-AN, Diglycosylceramide</td>
</tr>
<tr>
<td>Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The major gangliosides from each type of cell were isolated by preparative thin layer chromatography and subjected to mild acid hydrolysis and neuraminidase hydrolysis as described under "Materials and Methods." The GSL and sialic acid products released on hydrolysis were identified by thin layer chromatography and comparison with standard compounds of known composition. From these observations and from the carbohydrate stoichiometry reported in Table V, a general structure for each of the gangliosides studied is proposed.
ing in chromatographic migration to diglycosylceramide and N-AN (Fig. 2A, Channel 2). Neuraminidase treatment of this ganglioside also yielded diglycosylceramide and N-AN (Fig. 2A, Channel 3). The GM3 species of 3T3-Py6 (Channel 4) when subjected to mild acid hydrolysis (Channel 5) and neuraminidase treatment (Channel 6), also yielded diglycosylceramide and N-AN. Similar results were obtained with the purified GM3 species of 3T3-SV-479 (Fig. 2B, Channel 1) treated with mild acid (Fig. 2B, Channel 2) or neuraminidase (Fig. 2C, Channel 2). A consistent observation throughout these various experiments was that the diglycosylceramide moiety liberated from the GM3 species migrated as a double band. This was particularly evident in Channels 2 and 3 of Fig. 2C.

The GM3 species of 3T3 cells was also subjected to mild acid and neuraminidase hydrolysis. Acid hydrolysis yielded products corresponding in chromatographic migration to tetraglycosylceramide and N-AN, and neuraminidase treatment produced bands corresponding in chromatographic migration to GM1 and N-AN. The results of these hydrolytic studies have been summarized in Table IV.

Gas-Liquid Chromatography Analyses of Sugar Moieties of Gangliosides of Normal and Virus-transformed 3T3 Cells—The carbohydrate composition of the principal gangliosides of 3T3, 3T3-SV-479, and 3T3-Py6 cells was determined by gas-liquid chromatography analyses of the trimethylsilyl ethers of the O-methyl glycosides. As shown in Fig. 3A an analysis of the ganglioside corresponding in chromatographic migration to GM1 derived from the normal 3T3 cells yielded the various derivatives of galactose, glucose, and N-AN. No significant amount of any other amino-sugar derivative was detected. As shown in Fig. 3B the analysis by gas-liquid chromatography of the GD1a species of 3T3 cells gave the various derivatives of galactose, glucose, galactosamine, and N-AN. The peaks corresponding to the different sugar derivatives were quantitated and the information obtained has been summarized in Table V.

Studies on Neutral GSLs of Normal and Virus-transformed Cells—The neutral GSL fractions of the normal 3T3 cells and the various transformed cells were prepared by silicic acid chromatography and subjected to thin layer chromatography. A representative chromatogram, shown in Fig. 4, revealed the principal GSLs of both normal and virus-transformed cells (Channels 1 to 6). These corresponded in chromatographic migration to standards of mono- and diglycosylceramide (Channel 7). Negligible amounts of more complex GSLs were detectable. A plasma membrane fraction of 3T3 cells (Channel 5) revealed the same pattern as that of the whole cells. No major differences in the pattern of GSLs from normal and transformed cells were evident from visual examination of stained chromatograms.

The two principal GSLs were purified by thin layer chromatography from 3T3, 3T3-SV-479, and 3T3-Py6 cells and their sugar moieties were analyzed by gas-liquid chromatography to

**Table V**

Results of gas-liquid chromatographic analyses of carbohydrate components of principal glycosphingolipids of normal and virus-transformed 3T3 cells

<table>
<thead>
<tr>
<th>Source of GSL analyzed and its chromatographic migration</th>
<th>Glucose</th>
<th>Galactose</th>
<th>Galactosamine</th>
<th>Sialic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3 Cells, GM1</td>
<td>0.94</td>
<td>1.00</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td>3T3 Cells, GM3</td>
<td>1.19</td>
<td>2.00</td>
<td>0.58</td>
<td>1.05</td>
</tr>
<tr>
<td>3T3 Cells, GD1a</td>
<td>1.12</td>
<td>2.00</td>
<td>0.97</td>
<td>2.07</td>
</tr>
<tr>
<td>3T3 Cells, MC1</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3 Cells, DC</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-SV-479 Cells, GM1</td>
<td>0.96</td>
<td>1.00</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>3T3-SV-479 Cells, MC1</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-Py6 Cells, GM1</td>
<td>0.84</td>
<td>1.00</td>
<td></td>
<td>1.08</td>
</tr>
<tr>
<td>3T3-Py6 Cells, MC1</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-Py6 Cells, DC</td>
<td>0.93</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* MC, monoglycosylceramide; DC, diglycosylceramide.
FIG. 4. Thin layer chromatogram of the neutral GSLs of normal and virus-transformed 3T3 cells and of plasma membrane fractions of these cells. Neutral GSLs of 3T3 cells (1), 3T3-SV-479 cells (2), plasma membrane fraction of 3T3 cells (3), plasma membrane fraction of 3T3-SV-479 cells (4), 3T3-Py6 cells (5), and 3T3-SV-CE66 cells (6). A mixture of human brain cerebroside and human kidney neutral GSL standards was applied in Channel 7. Four-tenths milligram of the neutral GSL fractions of each cell type and membrane fraction was separated by thin layer chromatography on Silica Gel G with chloroform-methanol-water (65:25:4, by volume) as the developing solvent. The GSLs were detected as blue bands by spraying the plate with aniline-diphenylamine reagent. CM, monoglycosylceramide; CD, diglycosylceramide; CT, triglycosylceramide; CT\', tetraglycosylceramide; G, ganglioside.

FIG. 6. Thin layer chromatogram of gangliosides from normal and virus-transformed 3T3 cells incubated with [1-\%]glucosamine. A, autoradiogram. Ganglioside fraction from human brain (1), 3T3 cells (2), 3T3-SV-479 cells (3), and 3T3-Py6 cells (4). B, same plate as shown in A except stained with the resorcin reagent to show the correspondence of radioactive zones to the chemically detected zones. Incubation of cells with [1-\%]glucosamine was performed as described under "Materials and Methods." Approximately 60 nmoles of LBSA were spotted in Channel 2 and 20 nmoles were spotted in Channels 3 and 4. The total radioactivity applied in each channel was approximately 18,000 dpm. Chromatographic conditions were as described in the legend of Fig. 1.

Fig. 7. Analysis by thin layer chromatography of the effect of neuraminidase treatment on the gangliosides of [\%]glucosamine-labeled 3T3 and 3T3-Py6 cells in suspension. A, autoradiogram of various [\%]glucosamine-labeled ganglioside fractions derived from cells harvested with PBS-EDTA. 1, 3T3 cells, 45 nmoles of LBSA applied; 2, 3T3 cells treated with neuraminidase, 45 nmoles of LBSA applied; 3, 3T3 cells, gangliosides extracted from 10 mg of total cell protein applied; 4, 3T3 cells treated with neuraminidase, gangliosides extracted from 10 mg of total cell protein applied; 5, 3T3-Py6 cells treated with neuraminidase, gangliosides extracted from 10 mg of total cell protein applied; 6, 3T3-Py6 cells, gangliosides extracted from 10 mg of total cell protein applied. B, same plate as in A but stained with resorcinol reagent to show correspondence of the radioactive zones with the chemically detected zones. The samples in Channels 1 to 6 are as described above; the sample in Channel 7 is the ganglioside fraction of human brain.

obtain the data shown in Table V. The monoglycosylceramide species from all three types of cells contained only glucose, and the diglycosylceramide species contained approximately equimolar amounts of glucose and galactose.

A limited number of quantitative analyses of the neutral GSL content of these three cells was performed, using mannitol as an internal standard. The results presented in Table VI provided quantitative confirmation for the qualitative data yielded by thin layer chromatography, which indicated that there were no marked differences in the amounts of the GSLs between the control and transformed cells studied. Tentative general structures for the neutral GSLs proposed in Table VI were based on the stoichiometric analyses reported in Table V.
An aliquot of mannitol was added to each sample as an internal standard. The individual GSLs were then methanolysed, the C₁₆:0, C₁₈:0, C₂₀:0, and C₂₂:0 normal fatty acids; and m/e 503, 531, 486, and 484 the presence of C₁₄:0, C₁₈:0, C₂₀:0, and C₂₂:0 hydroxy fatty acids, respectively (49).

...the presence of C₁₆:0, C₁₈:0, C₂₀:0, and C₂₂:0 normal fatty acids; and m/e 503, 531, 486, and 484 the presence of C₁₄:0, C₁₈:0, C₂₀:0, and C₂₂:0 hydroxy fatty acids, respectively (49).

The results are averages of duplicate analyses on two batches of each type of cell. A tentative structure for each of the GSLs analyzed is also proposed on the basis of the individual sugar stoichiometry reported in Table V.

### Table VI
**Neutral glycosphingolipid content of normal and transformed 3T3 cells**

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Chromatographic migration of GSL analyzed</th>
<th>GSL content (nanomoles/mg protein)</th>
<th>Tentative structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3</td>
<td>MC*</td>
<td>1.06</td>
<td>Cer-Glu</td>
</tr>
<tr>
<td>3T3</td>
<td>DC</td>
<td>1.14</td>
<td>Cer-Glu-Gal</td>
</tr>
<tr>
<td>3T3-SV-479</td>
<td>MC</td>
<td>2.00</td>
<td>Cer-Glu</td>
</tr>
<tr>
<td>3T3-SV-479</td>
<td>DC</td>
<td>0.89</td>
<td>Cer-Glu-Gal</td>
</tr>
<tr>
<td>3T3-Py6</td>
<td>MC</td>
<td>1.13</td>
<td>Cer-Glu-Gal</td>
</tr>
<tr>
<td>3T3-Py6</td>
<td>DC</td>
<td>1.21</td>
<td>Cer-Glu-Gal</td>
</tr>
</tbody>
</table>

*Expressed as nanomoles of glucose.

**MC**, monoglycosylelemame; **DC**, diglycosylelemame.

### GSLs of Control and Transformed Cells

The methyl esters of the fatty acids obtained during the methanolysis procedure used for the analysis of the sugar moieties of the GSLs were examined by gas-liquid chromatography. The results, shown in Table VII, revealed that a relatively wide spectrum of fatty acids, both saturated and unsaturated, was present in the compounds studied. Palmitic (16:0), stearic (18:0) and oleic (18:1) acids were the principal fatty acids detected in both the gangliosides and neutral GSLs; linoleic (18:2) was also prominent, particularly in the monoglycosylelemame species of the various cells. Whereas the amounts of palmitic and stearic acid were generally appreciably greater in the gangliosides and neutral GSLs, the amount of oleic acid showed the opposite trend, being appreciably lower in amount in the gangliosides than in the neutral GSLs.

**Mass Spectrometric Analysis of GM₃ Species**

In order to obtain additional structural information on the GM₃ species of 3T3 and 3T3-SV-479 cells, studies were carried out using mass spectrometric analysis. The main features suggested from the fragmentation pattern of the GM₃ species of normal 3T3 cells (see Fig. 5) were the following (49): (a) the predominance of N-AN (represented in prominent peaks at m/e 180 and 178) over N-GN (minor peaks at m/e 274 and 261); (b) the presence of C₁₄ sphingosine (peak m/e 311) and C₁₈ sphinganine (peak m/e 313); (c) the virtual absence of eicospinosine (negligible peak at m/e 389); (d) the presence of fragments derived from hydroxyfatty acids (e.g., minor peaks between m/e 327 and 411). Mass spectrometric analysis was also performed on the GM₃ species from 3T3-SV-479 cells. Minor differences were observed in the peaks due to the derivatives of hydroxyfatty acids, but otherwise the fragmentation pattern was identical with that obtained from the GM₃ species of normal 3T3 cells.

**Incorporation of [1-¹⁴C] Glucosamine into Gangliosides of Normal and Virus-transformed 3T3 Cells**

The capacity of 3T3, 3T3-SV-479, and 3T3-Py6 cells to synthesize gangliosides was investigated by incubation of these cells with radioactive glucosamine, previously shown to be a useful precursor for demonstrating ganglioside biosynthesis in cultured mouse cells (26). Normal and virus-transformed 3T3 cell, incubated with [¹⁴C] glucosamine for approximately 24 hours, were analyzed for the distribution of radioactivity derived from this isotope in various cell fractions, as noted in Table VIII. Approximately 75 to 85% of the total [¹⁴C] glucosamine taken up by the normal and transformed cells was present in the nonlipid residue. The remainder was distributed in the lipid fraction, particularly in the upper phase of the Folch extract. When the upper phase derived from normal 3T3 cells was subjected to thin layer chromatography to separate individual gangliosides and the radioactivity in each of the gangliosides was determined, significant radioactivity was detected in GM₁, GM₂, GM₃, and GD₁. In contrast, similar analyses on material extracted from 3T3-SV-479 and 3T3-Py6 cells revealed that the majority of the radioactivity was present in the GM₃ species.

The distribution of radioactivity derived from [¹⁴C] glucosamine in the ganglioside fractions of normal and transformed 3T3 cells was also investigated by performing autoradiography of the thin layer chromatograms. As shown in Fig. 6A, considerably less radioactivity derived from [¹⁴C] glucosamine was present in the areas of the chromatograms behind GM₁ in the ganglioside fractions of 3T3-SV-479 (Channel 3) and 3T3-Py6 (Channel 4) cells as compared with the normal cells. The autoradiographic technique was in fact more sensitive than the resorcinol method of detection, in that distinct radioactive zones were detectable behind GM₁ in the ganglioside fractions of the transformed cells (Fig. 6A, Channels 3 and 4) where none were detected in visual...
Fatty acid composition of principal gangliosides and neutral glycosphingolipids of normal and virus-transformed 3T3 cells

The fatty acid composition of the purified lipids was determined by gas chromatographic analyses of their methyl esters prepared by methanolation. The results are averages of duplicate analyses of purified glycosphingolipids from each of two batches of cells.

<table>
<thead>
<tr>
<th>Major fatty acids</th>
<th>JT3 Cells</th>
<th>JT3-SV-179 Cells</th>
<th>JT3-Py6 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MC*</td>
<td>DC</td>
<td>GMs</td>
</tr>
<tr>
<td>Myristic (14:0)</td>
<td>3.5</td>
<td>2.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Palmitic (16:0)</td>
<td>19.9</td>
<td>21.8</td>
<td>39.2</td>
</tr>
<tr>
<td>Palmitoleic (16:1)</td>
<td>5.6</td>
<td>3.1</td>
<td>3.5</td>
</tr>
<tr>
<td>Stearic (18:0)</td>
<td>7.1</td>
<td>7.6</td>
<td>27.1</td>
</tr>
<tr>
<td>Oleic (18:1)</td>
<td>33.9</td>
<td>44.8</td>
<td>11.7</td>
</tr>
<tr>
<td>Linoleic (18:2)</td>
<td>13.8</td>
<td>4.3</td>
<td>Trace</td>
</tr>
<tr>
<td>Arachidonic (20:4)</td>
<td>1.8</td>
<td>2.6</td>
<td>3.0</td>
</tr>
<tr>
<td>Behenic (22:0)</td>
<td>6.6</td>
<td>3.3</td>
<td>12.2</td>
</tr>
<tr>
<td>Lignoceric (24:0)</td>
<td>7.8</td>
<td>10.4</td>
<td></td>
</tr>
</tbody>
</table>

* MC, monoglycosyleramid; DC, diglycosyleramid.

A fatty acids are designated by chain length:number of double bonds.

The principal gangliosides of the normal 3T3 cells used in this study corresponded in chromatographic migration to GM₃, GM₁, and GD₁a, (Fig. 1 and Table III). Two of these gangliosides, corresponding in migration to GM₁ and GD₁a, were chosen for relatively detailed structural analyses. Hydrolytic studies and analyses by gas-liquid chromatography indicated that these two compounds had general structures compatible with those indicated in Table IV, and were in fact GM₂ and GD₁a species. The ganglioside composition for normal 3T3 cells described here agreed with that observed by Brady and Mora (9) using analyses by thin layer chromatography, and with that reported recently by Dijong et al. (27) on the basis of analyses by gas-liquid chromatography. The present studies extended the observations of these workers by identifying the sialic acid species present in GM₂ and GD₁a, describing the fatty acid composition of these compounds and reporting the mass-spectrometric analysis of GM₂. Analyses by thin layer chromatography indicated that N-AN was virtually the sole sialic acid species present in both GM₂ and GD₁a. In addition, both of these purified gangliosides were found to be susceptible to V. cholerae neuraminidase, the GSL products being compounds corresponding in chromatographic migration to dihexosylceramide and GM₁, respectively. These observations concurred with previous studies on the action of neuraminidase on GM₂ and GD₁a (51). The fatty acid data also added to the structural information available on 3T3 cell gangliosides, and revealed that the principal gangliosides of these cells contained a wide range of fatty acids. Weinstein et al. (23) have demonstrated that the principal gangliosides of cultured L-cells exhibit a wide range of fatty acid composition, in contrast to the restricted pattern observed in brain gangliosides. The mass-spectrometric analysis of the GM₂ species of normal 3T3 cells, interpreted largely after Dawson and Sweeley (49), confirmed the predominance of N-AN and suggested the presence of both C₁₈ sphingosine and C₁₈ sphinganine and a variety of hydroxy fatty acids. The observation that, in optimal chromatographic separations, GM₂ migrated as a double band is thus possibly explained by the presence of hydroxy fatty acids in the slower migrating band (52). It is apparent that mass-spectrometric analysis is an extremely powerful tool for the analysis of GSLs of cultured cells, particularly in view of its applicability.
of the two neutral GSLs studied here were in general similar to those of the gangliosides and are thus compatible with a fraction, at least, of these compounds being precursors of the gangliosides (53). Both neutral GSLs migrated as double bands, presumably due to the presence of hydroxy fatty acids. The virtual absence of trichosylosceramide (galactosylgalactosylglycosylerosceramide) in the normal 3T3 cells is of particular interest as Kijimoto and Hakomori (16) have observed that the activity of the glycolipid glycosyltransferase converting galactosylerosceramide to galactosylgalactosylglycosylerosceramide is increased in hamster kidney cells in confluent cultures. The present observations, indicating a virtual absence of trichosylosceramide in confluent normal 3T3 cultures, might suggest that the above process does not operate significantly in these cells.

Plasma membrane preparations obtained from 3T3 cells were found to be enriched with respect to gangliosides (expressed as moles of L18SA per mg of protein) as compared with analyses on whole cells. The purification observed was comparable to the 8- to 5-fold enrichment reported by Kleink and Choppin (22) and by Weinstein et al. (23) for cultured monkey cells and for mouse L-cells, respectively. It was, however, less than the 10-fold enrichment obtained by Renkonen et al. (24) with BHK-21 cells. Like Kleink and Choppin (22), we have observed no marked differences in the distribution of individual gangliosides in plasma membrane preparations as compared with whole cell extracts. In contrast, Weinstein et al. (29) found that one monosialoganglioside present in whole cells was not detected in extracts of isolated L-cell surface membranes. The relatively modest enrichment of gangliosides in the surface membrane fractions as compared with the more marked enrichment of Na+K+ ATPase (10- to 20-fold) is somewhat puzzling. It is possible that subfractions of plasma membrane exist with differing enzymic and lipid content. Another possibility, not yet excluded, would be that gangliosides are predominantly located in another intracellular fraction (e.g. Golgi apparatus) that contaminates the surface membrane preparation. Complete cell balance studies are required to settle this issue. Both neutral GSLs found in normal 3T3 cells were detected in purified plasma membrane preparations. Their pattern in the plasma membrane (as studied by thin layer chromatography) appeared similar to that of the whole cell extracts (Fig. 5), but the precise amount of these compounds in the plasma membrane preparations was not studied.

Isolated GM1 and GD1a from 3T3 cells were found to be susceptible to V. cholerae neuraminidase. This made it possible to examine the susceptibility to this enzyme of these compounds in their membrane-bound state in 3T3 cells. As shown in Fig. 7, a 3-hour treatment of 3T3 and 3T3-Py6 cells in suspension with this enzyme (at neutral pH) resulted in a significant decrease in the amount of GM1 present in these cells. In the case of 3T3 cells, GD1a was partly converted to a GM1 species. Similar results were obtained when these cells were treated in situ with neuraminidase. These observations do not permit precise localization of the membrane-bound gangliosides since the neuraminidase may have penetrated the outer periphery of the cells (54). It has been established that neuraminidase can liberate the sialic acid moieties of the gangliosides of the red cell membrane (55). It would appear from the present study that it can also release considerable amounts of L18SA from morphologically intact 3T3 cells, both in suspension and in monolayer, and at least part of this is presumably derived from these ganglio-

Table VIII

Distribution of radioactivity from L-3H]glucosamine in lipid and nonlipid fractions and individual ganglioside moieties of normal and virus-transformed 3T3 cells

<table>
<thead>
<tr>
<th>Percentage of total cellular radioactivity</th>
<th>3T3 Cells</th>
<th>3T3-SV-479 Cells</th>
<th>3T3 Py6 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> Total cells</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Nonlipid residue</td>
<td>74.6 ± 7.7</td>
<td>84.5 ± 1.1</td>
<td>79.6 ± 2.9</td>
</tr>
<tr>
<td>Lower phase</td>
<td>4.1 ± 0.4</td>
<td>0.3 ± 0.1</td>
<td>2.2 ± 1.3</td>
</tr>
<tr>
<td>Upper phase</td>
<td>21.3 ± 7.3</td>
<td>15.2 ± 1.1</td>
<td>18.2 ± 2.1</td>
</tr>
<tr>
<td><strong>B</strong> Upper phase</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>GM1</td>
<td>36.9 ± 4.3</td>
<td>44.2 ± 3.7</td>
<td>42.2 ± 2.6</td>
</tr>
<tr>
<td>GM2</td>
<td>7.9 ± 0.4</td>
<td>3.3 ± 1.4</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>GM1</td>
<td>5.0 ± 0.5</td>
<td>1.9 ± 1.0</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>GD1a</td>
<td>16.7 ± 4.0</td>
<td>2.4 ± 0.0</td>
<td>1.8 ± 1.0</td>
</tr>
<tr>
<td>GD1b</td>
<td>2.5 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Origin to GT</td>
<td>13.3 ± 3.9</td>
<td>25.5 ± 15.1</td>
<td>39.6 ± 4.7</td>
</tr>
<tr>
<td>Average recovery</td>
<td>82.3</td>
<td>77.3</td>
<td>77.8</td>
</tr>
</tbody>
</table>

Results are means of duplicate analyses of three experiments with standard deviations calculated. In an experiment, to each of 5 bottles containing approximately 1.6 X 10^6 cells in 20 ml of CMRL 1066 medium-7.5% fetal calf serum, was added 2 μCi of L-3H]glucosamine. The cells were then incubated at 37°C for 24 hours, harvested, washed twice with PBS, and an aliquot taken for determination of total cell radioactivity. 1.5 ± 0.5%, 3.9 ± 0.5%, and 3.5 ± 0.4% of the [3H]glucosamine added to the medium was taken up by the 3T3, 3T3-SV-479, and 3T3-Py6 cells, respectively. The remaining cells were extracted to obtain the data shown under A relative to the distribution of radioactivity in the nonlipid residue and the lower and upper phases of the Folch extract. To obtain the data shown under B, aliquots of the upper phase were subjected to thin layer chromatography to separate individual gangliosides, and the radioactivity in the various areas of the chromatograms was determined. The total radioactivity in the cells when expressed on the basis of disintegrations per min per mg of total cell protein was as follows: 3T3 cells, 1.6 X 10^4; 3T3-SV-479 cells, 7.9 X 10^4; 3T3-Py6 cells, 3.6 X 10^4. The total disintegrations per min present in the upper phase expressed per mg of total cell protein averaged 3.4 X 10^4 for 3T3 cells, 1.2 X 10^4 for 3T3-SV-479 cells, and 6.5 X 10^3 for 3T3-Py6 cells.
sides that are present in the plasma membrane. We did not examine the metabolic capacities, plating efficiencies, or ultrastructural appearance of the cells inoculated with neuraminidase, and the criteria used for evaluating cell integrity were crude (light microscopy and trypan blue exclusion). Nevertheless, these studies would suggest that the possibility that sialic acid attached to ganglioside is being removed (as well as, or other than, glycoprotein-linked sialic acid) must at least be considered in interpreting the effects on intact cells, for example certain tumor cells, of treatment with neuraminidase.

Brady and Mora (9) have previously observed that certain derivatives of SV40- and polyoma-transformed 3T3 cells show a marked simplification of ganglioside composition in comparison with normal 3T3 cells. The simplification noted was analogous to that observed by Hakomori and Murakami (7) in polyoma-transformed BHK cells, but differed in the specific GSLs affected. The transformed 3T3 cells studied by Brady and Mora lacked gangliosides more complex than GM3 and also revealed a marked diminution in the activity of the UDP-galNAc transferase converting GM3 to GM2 (17). The 3T3-SV-479 and 3T3-Py6 isolates examined in the present study were thus similar in ganglioside profile to the virally transformed 3T3 cells studied by Brady and Mora in that both of these isolates contained principally GM2, as determined by chemical and radiochromatographic analyses. In contrast, clonal isolates 3T3-SV-A26 and 3T3-SV-CF56 exhibited a complex pattern of gangliosides, qualitatively similar to that of the control 3T3 cells, but showing an increase in the amount of the ganglioside GD1a. Mora et al. (17) have noted that certain “flat-type” revertant 3T3 cells contain a complex pattern of gangliosides. It seems unlikely that 3T3-SV-A26 and 3T3-SV-CF56 were revertant as they exhibited a phenotype similar to that of 3T3-SV-479 and 3T3-Py6 cells. That they contained T antigen, grew on irradiated 3T3 cells, supported growth of BEV virus, and were agglutinated by low concentrations of concanavalin A (Table I). 3T3-SV-CF56 cells were tumorigenic, although no tumors were obtained by 12 months after inoculation with 3T3-SV-A26 cells. We have therefore concluded that different isolates of SV40-transformed 3T3 cells show clonal variation with respect to ganglioside composition. What is the basis of this apparent clonal variation? It may reflect the selection of pre-existent clonal variants with differing GSL profiles from the population of cells originally transformed by the oncogenic virus. It may arise from a separation and subsequent selection of mutant cells, again with differing GSL profiles, as a result of infection by this oncogenic virus (cf. 57). Another possibility is that the expression of the genes controlling ganglioside synthesis may be affected by the site at which viral genome is integrated within the chromosome of the transformed cell (17).

Variation in the GSL profile of clonal isolates has now been described for hamster cells transformed by hamster sarcoma virus (58), mouse cells transformed by SV40 virus, and radiation-induced tumorigenic mouse adrenocortical cells (59). Variations in the glycoprotein profile of surface membrane fractions of the isolates studied here have also been observed (33).

Several other points with regard to the findings made in this investigation on the GSL profile and content of the virally transformed cells studied merit brief comment. First, no correlation was evident between the complexity of the GSL pattern of the cells studied and their saturation density (Table I). In this regard it is relevant to note that addition of cyclic adenosine 3',5'-monophosphate to 3T3-Py6 cells, in concentrations sufficient to restore inhibition of cell multiplication (60), did not affect the GSL pattern of these cells. In particular, no evidence of a “turn-on” of the synthesis of the complex gangliosides that are markedly diminished in these cells as compared with control 3T3 cells was detected. These observations might appear to support the view that there may be little correlation between complexity of ganglioside pattern and saturation density.

A second point is that the general structural features of the GM2 and neutral GSL species of 3T3-SV-479 and 3T3-Py6 cells appeared similar to those of the corresponding glycolipids of the control 3T3 cells. A possible difference in the hydroxy fatty acid composition of the GM2 species was observed by mass spectrometric analysis, but separate analyses of the hydroxy fatty acid composition of the normal and transformed cells will be required to assess the significance of this. Another point of interest is that, despite the variation in GSL pattern observed in the various transformed cells studied, all of these cells were more sensitive to agglutination by concanavalin A than were the normal 3T3 cells. The increased sensitivity of transformed cells to agglutination by concanavalin A appears well documented (cf. 61). This observation might suggest that surface GSLs do not play a major role in the events that determine cell agglutination by this lectin.

The studies described here, as well as those summarized earlier, indicate that a derangement of GSL metabolism occurs in at least certain cells transformed by SV40 or polyoma virus. A critical question, as yet to be answered unequivocally, is how early do these changes in GSL metabolism occur during the process of virus transformation? Studies that would answer this question with regard to cells transformed by SV40 or polyoma virus have yet to be reported. Early alterations of GSL metabolism have been observed in chicken cells transformed by Rous sarcoma virus (10). However, Warren et al. (62) did not observe early alterations in GSL metabolism in chicken cells transformed by a temperature-sensitive mutant of this virus. Mouse embryo secondary cultures, several days after transformation by murine sarcoma virus, did not show significant alterations of GSL metabolism. A definitive answer to the above question must await the provision of additional evidence. However, it is meanwhile apparent that caution must be employed in the interpretation of the significance of alterations of GSL composition in relatively long established lines of transformed cells, because of the possible variation of this composition in different clonal isolates. Further studies are in progress in our laboratories to explore the factors regulating GSL synthesis in control and transformed cultured cells, and also to assess the significance of the differences in GSL patterns observed in clonal isolates of transformed cells.

Note Added in Proof.—Several clonal isolates of cultured mouse neuroblastoma cells have also been observed to differ in their ganglioside composition (63).

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Studies on the Glycosphingolipids of Normal and Virally Transformed 3T3 Mouse Fibroblasts
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