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# Lipid Composition of PC12 Pheochromocytoma Cells: Characterization of Globoside as a Major Neutral Glycolipid†

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ABSTRACT: We have studied the lipid composition of PC12 pheochromocytoma cells cultured in the presence and absence of nerve growth factor (NGF). Neutral and acidic lipid fractions were isolated by column chromatography on DEAE-Sephadex and analyzed by high-performance thin-layer chromatography (HPTLC). The total lipid concentration was approximately 220 μg/mg of protein, and the concentration of neutral glycolipids was 1.6–1.8 μg/mg of protein for both NGF-treated and untreated cells. The neutral glycolipid fraction contained a major component, which accounted for approximately 80% of the total and which was characterized as globoside on the basis of HPTLC mobility, carbohydrate analysis, fast atom bombardment mass spectrometry, and mild acid hydrolysis. The major fatty acids of globoside were C16:0 (10%), C18:0 (16%), C22:0 (23%), C24:1 (17%), and C24:0 (24%). C18 sphingenine accounted for almost all of the long-chain bases. The other neutral glycolipids were tentatively identified as glucosylceramide (15%), lactosylceramide (4%), and globotriosylceramide (4.5%). The concentration of ganglioside sialic acid was approximately 0.34 and 0.18 μg/mg of protein for cells grown in the presence and absence of NGF, respectively. Although there was an increase in ganglioside concentration in NGF-treated cells, NGF did not produce any differential effects on the relative proportions of the individual gangliosides. Several of the gangliosides appear to contain fucose, and one of these was tentatively identified as fucosyl-$GM_1$. Brain-type gangliosides of the ganglio series were also detected by an HPTLC–immunostaining method. However, the fatty acid and long chain base compositions of PC12 cell gangliosides (and their TLC mobility) differ from those of brain gangliosides. The major lipids of PC12 cells, which were not significantly affected by NGF treatment, were phosphatidylcholine (40%), phosphatidylethanolamine (22%), and cholesterol (13%).

The PC12 cells, a clonal line of rat adrenal medullary pheochromocytoma cells, display many properties associated with normal adrenal chromaffin cells and undergo striking morphological and physiological changes in response to nerve growth factor (NGF),[1] which converts them into a sympathetic neuron-like phenotype [for a review, see Greene and Tischler (1982)]. Glycosphingolipids have been shown to be located almost exclusively in the outer leaflet of plasma membranes and have been implicated in many cell surface phenomena (Hakomori, 1981). In spite of the utility of PC12 cells for the study of various types of cell surface events, little is known concerning their complex glycosphingolipid composition. It was previously reported that NGF treatment produces an almost 3-fold increase in the incorporation of labeled gluco-

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[1] Abbreviations: Cer, ceramide (*N*-acylsphingoid); GlcCer, Glcβ1-1Cer; LacCer, Galβ1-4Glcβ1-1Cer; $Gg_3$ (asialo-$G_{M2}$), GalNAcβ1-4Galβ1-4Glcβ1-1Cer; $Gg_4$ (asialo-$G_{M1}$), Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; $Gb_3$, Galα1-4Galβ1-4Glcβ1-1Cer; $Gb_4$ (globoside), GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer; $Gb_5$, GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer; NeuAc, *N*-acetylneuraminic acid; $G_{M3}$, NeuAcα2-3Galβ1-4Glcβ1-1Cer; $G_{M2}$, GalNAcβ1-4Gal(3-2αNeuAc)β1-4Glcβ1-1Cer; $G_{M1}$, Galβ1-3GalNAcβ1-4Gal(3-2αNeuAc)β1-4Glcβ1-1Cer; $G_{D1a}$, NeuAcα2-3Galβ1-3GalNAcβ1-4Gal(3-2αNeuAc)β1-4Glcβ1-1Cer; $G_{D1b}$, Galβ1-3GalNAcβ1-4Gal(3-2αNeuAc8-2αNeuAc)β1-4Glcβ1-1Cer; $G_{D3}$, NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1Cer; $G_{D2}$, GalNAcβ1-4Gal(3-2αNeuAc8-2αNeuAc)β1-4Glcβ1-1Cer; fucosyl-$G_{M1}$ (or $G_{M1}$-Fuc), Fucα1-2Galβ1-3GalNAcβ1-4Gal(3-2αNeuAc)β1-4Glcβ1-1Cer; $G_{T1b}$, NeuAcα2-3Galβ1-3GalNAcβ1-4Gal(3-2αNeuAc8-2αNeuAc)β1-4Glcβ1-1Cer; $G_{Q1b}$, NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-4Gal(3-2αNeuAc8-2αNeuAc)β1-4Glcβ1-1Cer; NGF, nerve growth factor; HPTLC, high-performance thin-layer chromatography; GLC, gas–liquid chromatography.

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samine into the hexosamine and sialic acid residues of PC12 cell gangliosides (Margolis et al., 1983) and that several monosialogangliosides are labeled when PC12 cells are cultured in the presence of L-[$^3$H]fucose (Margolis et al., 1984). More recently, Schwarting et al. (1986) confirmed these findings and reported the presence of two fucosylated ganglio-series gangliosides, partially characterized as fucosyl-$G_{M2}$ and fucosyl-$G_{D2}$. However, since little information was available concerning the concentrations of gangliosides and related lipids in PC12 cells and because of questions concerning the structural relationships of PC12 cell glycolipids to those in other types of nervous tissue, we have investigated these questions in detail. We found that globoside is the major glycosphingolipid in both NGF-treated and untreated PC12 cells. Our studies also revealed that PC12 cells contain many ganglio-series gangliosides and support the previous conclusion (Margolis et al., 1983) that NGF treatment increases the concentration of PC12 cell gangliosides without differentially affecting the proportions of the individual ganglioside species, including several that contain fucose. Finally, in agreement with the findings of Traynor et al. (1982), we detected little effect of long-term NGF treatment on the non-glycolipid composition of PC12 cells. A preliminary account of this work has been reported (Yu et al., 1985).

## Experimental Procedures

*Materials.* High-performance TLC plates (nano-plates, 10 × 20 cm) were purchased from E. Merck, Darmstadt, FGR. DEAE-Sephadex A-25 and Iatrobeads were suppled respectively by Pharmacia Fine Chemicals, Uppsala, Sweden, and Iatron, Tokyo, Japan. Fucosyl-$G_{M1}$ was prepared from pig adipose tissue and was kindly supplied by Dr. M. Ohashi, Ochanomizu University, Tokyo, Japan. Other glycolipid standards were prepared in our laboratories according to published methods (Ando et al., 1977; Ariga et al., 1980; Kasai et al., 1982). Rabbit anti-asialo-$G_{M1}$ serum was prepared and characterized in our laboratory as described previously (Jacobson et al., 1982; Saito et al., 1985b). *Arthrobacter ureafaciens* neuraminidase and $^{125}$I-labeled staphylococcal protein A were supplied by Calbiochem-Behring, San Diego, CA, and New England Nuclear, Waltham, MA, respectively. *N*-Methylbis(trifluoroacetamide) (MBTFA) was purchased from Pierce Chemical Co., Rockford, IL. Other chemicals and reagents were of analytical grades and were obtained from commercial sources.

*Cell Culture.* PC12 cells were grown on collagen-coated culture dishes as previously described (Green & Tischler, 1976, 1982). Some cultures were treated for 2 or more weeks with 50 ng/mL mouse submaxillary NGF prepared according to Mobley et al. (1976). Batches of cells (from 20 to 40 culture dishes of 100- or 150-mm diameter) were rinsed free of medium with phosphate-buffered saline and harvested by scraping with a rubber policeman. This material was stored at −20 °C until used for analysis. Small aliquots were set aside for determination of total protein.

*Lipid Isolation.* The isolation procedures for the total lipids, and the neutral and acidic glycolipids, were described previously (Ando et al., 1977; Ariga et al., 1980; Macala et al., 1983; Sekine et al., 1984). Briefly, total lipids were extracted with chloroform–methanol (2:1 and 1:1 v/v) and chloroform–methanol–water (30:60:8 v/v) and filtered. The combined extracts were then applied to a DEAE-Sephadex A-25 column (acetate form, 5-mL bed volume), which was further eluted with 50 mL of chloroform–methanol–water (30:60:8 v/v). The neutral lipids were eluted in this fraction. Acidic lipids were then eluted with 50 mL of chloroform–methanol–0.8 M sodium acetate (30:60:8 v/v). A portion of the neutral and acidic lipid fractions was set aside for neutral glycolipid and ganglioside analysis, respectively, and the remainder was processed for analysis of total lipids (Macala et al., 1983).

*Comparative Study of Glycolipids.* Neutral glycolipids were isolated as previously described (Ariga et al., 1980), with slight modifications as follows. A part of the neutral lipid fraction was evaporated to dryness, dissolved in 2 mL of 0.2 N NaOH in methanol, and incubated at 40 °C for 2 h. After neutralization with 4 N acetic acid in methanol, the sample was partitioned according to Folch et al. (1957). The lower phase was evaporated, and the residue was applied to an Iatrobeads column (0.75 g, 3-mL bed volume). Cholesterol, fatty acids, etc. were removed by eluting with 15 mL of chloroform, and the neutral glycolipids were eluted with 7.5 mL each of 4:1 (v/v) and 3:2 (v/v) chloroform–methanol. The glycolipid fractions were combined and evaporated to dryness. An aliquot of this glycolipid fraction was examined by HPTLC with two different solvent systems: (A) chloroform–methanol–water (65:35:8 v/v) and (B) chloroform–methanol–2.5 N ammonia (60:40:10 v/v). The neutral glycolipids were visualized by spraying with 0.5% α-naphthol in 50% sulfuric acid and heating at 95 °C and then scanned at 580 nm (reference wavelength 730 nm) with a Shimadzu CS-910 densitometer.

Quantitation of the major neutral glycolipids was carried out by gas–liquid chromatography (GLC) of the trimethylsilyl derivatives of the individual sugar constituents (Vance & Sweeley, 1967), with myoinositol as an internal standard. After extraction of the fatty acid methyl esters with *n*-hexane, the methanolic layer was evaporated under nitrogen and dried under vacuum. The residue was trimethylsilylated with hexamethyldisilazane–trimethylsilane–pyridine (2:1:5 v/v), and an aliquot of this reaction mixture was injected onto a 3% OV-1 column maintained at 175 °C. Standard mixtures in various proportions from 5 to 30 μg of pure globoside (from pig erythrocyte membranes) containing 5 μg of myoinositol as an internal standard were also analyzed.

Gangliosides were isolated according to Ando and Yu (1977). Briefly, a portion of the acidic lipid fraction was evaporated to dryness under $N_2$ and vacuum, dissolved in 0.5 mL of 0.1 N NaOH, and incubated at 37 °C for 1 h. After neutralization with 1 N acetic acid, the sample was desalted on a 20 mL bed volume Sephadex G-50 column. Following lyophilization, sulfatides and fatty acids were removed by chromatography on a 2 mL bed volume Iatrobeads column to yield a ganglioside fraction. The sialic acid content of the gangliosides was then determined by GLC (Yu & Ledeen, 1970).

*Characterization of the Major Neutral Glycolipid (Globoside) and Gangliosides.* In order to characterize the major neutral glycolipid and the gangliosides, the total lipids were extracted from a large batch of PC12 cells (corresponding to 754 mg of protein). The lipid extract was dissolved in a small amount of methanol, and 20 mL of distilled water was added. After sonication it was dialyzed against 5 L of distilled water for 2 days. The retentate was evaporated to dryness under vacuum, and the residue, 170 mg, was dissolved in 10 mL of chloroform–methanol–water (30:60:8 v/v) and applied to a DEAE-Sephadex A-25 column (45-mL bed volume, 1.5 × 25 cm). The neutral glycolipid fraction was eluted with 50 mL of chloroform–methanol–water (30:60:8 v/v) and 100 mL of methanol. The acidic lipid fraction containing gangliosides was eluted stepwise with 150 mL each of 0.05 and 0.1 M sodium acetate in methanol to yield mono- and disialoganglioside fractions, respectively, and 200 mL of each of 0.2

and 0.3 M sodium acetate in methanol to yield a polysialoganglioside fraction.

(*1*) *Neutral Glycolipids.* The neutral lipid fraction thus obtained was subjected to mild alkali treatment as described above. The residue was dissolved in 0.5 mL of chloroform and applied to an Iatrobeads column (10 g, 1 × 40 cm). The glycolipids were fractionated and purified by stepwise elution with 50 mL each of chloroform–methanol (4:1, 1:1, and 1:3 v/v) and methanol. A major glycolipid was recovered in the chloroform–methanol (1:1 v/v) fraction.

The neutral sugar, amino sugar, and long chain base compositions of the isolated major glycolipid were analyzed by GLC of their trifluoracetate derivatives, and the fatty acid composition was analyzed by GLC of their methyl esters. The sample, 100 μg, was methanolyzed for 16 h at 75 °C with 200 μL of 3% methanolic HCl. Fatty acid methyl esters were extracted with *n*-hexane and analyzed on a 3% OV-1 column maintained at 205 °C. α-Hydroxy fatty acid methyl esters were further converted to their trimethylsilyl derivatives and analyzed on the same column maintained at 215 °C. The lower methanolic layer, containing methyl glycosides, was evaporated under nitrogen and dried under vacuum. The residue was trifluoroacetylated with 20 μL of 33% MBTFA [*N*-methylbis(trifluoroacetamide)] in pyridine for 10 min at room temperature. An aliquot of the reaction mixture was injected onto a 3% SP-2401 and 0.5% OV-225 column, temperature programmed at 3 deg/min from 140 to 230 °C (Ando & Yu, 1977). The long chain base composition was separately analyzed by the procedure of Gaver and Sweeley (1965).

About 100 μg of the isolated major glycolipid was subjected to mild acid hydrolysis with 0.5 mL of 0.1 N hydrochloric acid at 100 °C for 30 min. The reaction mixture was evaporated under nitrogen. The residue was dissolved in 0.5 mL of chloroform–methanol (2:1 v/v), and 0.1 mL of distilled water was added. The upper aqueous layer was removed, and the lower chloroform layer was washed twice with Folch's theoretical upper phase and then evaporated to dryness under nitrogen. The glycolipid hydrolysate was examined by HPTLC in solvent system A.

Fast atom bombardment (FAB) mass spectrometry of the isolated glycolipid was performed as follows. The sample, 50 μg, was dissolved in 10 μL of chloroform–methanol (2:1 v/v), and then 2 μL of triethanolamine–tetramethylurea (1:1 v/v) was added. The solvent mixture (about 1 μL) was applied to a stainless steel sample holder (1 × 4 mm) and analyzed on a negative ion FAB mass spectrometer (JMS HX 110, JEOL, Tokyo) equipped with a FAB ion source and JMA-3500 computer system (JEOL, Tokyo). Xenon gas was used at a 6-kV neutral beam (Arita et al., 1984).

(*2*) *Gangliosides.* The carbohydrate and fatty acid composotions of the gangliosides were analyzed by GLC of the trifluoroacetyl and trimethylsilyl derivatives, respectively, as described above. To detect the asialo-$GM_1$ (gangliotetraose) structure, the various ganglioside fractions obtained by DEAE-Sephadex column chromatography were examined by an HPTLC immunostaining method (Saito et al., 1985a,b). The fractions were chromatographed with chloroform–methanol–0.2% $CaCl_2 \cdot 2H_2O$ (55:45:10 v/v) (solvent C). The plate was dried under vacuum and immersed in 0.4% poly(isobutyl methacrylate) in *n*-hexane for 1 min. After drying, the chromatogram was incubated with neuraminidase from *Arthrobacter ureafaciens* (40 milliunits/mL in 0.1 M sodium acetate buffer, pH 4.8) for 2 h at room temperature, washed with 0.3% gelatin in phosphate-buffered saline (gelatin–PBS, pH 7.3), and dried in air. The plate was then incubated with a rabbit polyclonal antibody to asialo-$GM_1$ for 1.5 h at room temperature, followed by washing and drying in air. It was then incubated with $^{125}$I-labeled staphylococcal protein A (4.9 × $10^5$ cpm/mL in 0.3% gelatin–PBS) for 2 h at room temperature. The chromatogram was exposed to X-ray film at −70 °C, and after exposure the gangliosides on the plate were visualized by spraying with resorcinol–HCl reagent.

Table I: Neutral Glycolipid Composition (%) of PC12 Cells[a]

| neutral glycolipid | −NGF | +NGF |
|---|---|---|
| GlcCer | 14.1 ± 1.9 | 10.7 |
| LacCer | 4.0 ± 0.5 | 2.5 |
| $Gb_3$ | 4.5 ± 0.7 | 6.2 |
| $Gb_4$ | 77.5 ± 1.0 | 80.5 |
| total (μg/mg of protein) | 1.82 ± 0.03 | 1.64 |

[a] Values represent average ± SEM, of three different samples.

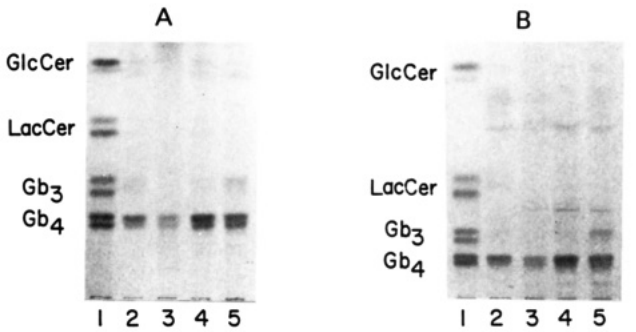


FIGURE 1: HPTLC of neutral glycolipids from PC12 cells: (lane 1) GlcCer from Gaucher's spleen and LacCer, $Gb_3$, and $Gb_4$ from pig erythrocyte membrane; (lanes 2–4) neutral glycolipid fractions from three different batches of PC12 cells; (lane 5) neutral glycolipid fraction from NGF-treated PC12 cells. Plate A was developed with solvent system A and plate B with solvent system B, as described in the text. The bands were visualized by spraying with α-naphthol/sulfuric acid reagent.

*Estimation of Each Lipid Class by HPLTC.* The isolated neutral and acidic lipids (excluding neutral and acidic glycolipids) were quantitated by high-performance thin-layer chromatography (HPTLC) according to an internal standard method (Macala et al., 1983).

## Results

*Neutral Glycolipid Composition of PC12 Cells.* The neutral glycolipid patterns of NGF-treated and untreated PC12 cells are shown in Table I and Figure 1. They contained one major glycolipid that cochromatographed with authentic globoside, obtained from pig erythrocyte membranes, on HPTLC in two different solvent systems. Other minor neutral glycolipids were tentatively identified as glucosylceramide, lactosylceramide and globotriosylceramide. The neutral glycolipid patterns of the NGF-treated and untreated cells did not differ significantly from each other with globoside representing approximately 80% of the total. The concentration of neutral glycolipids was also similar in cells grown in the presence or absence of NGF (Table I).

The concentration of the major neutral glycolipid isolated from a large-scale preparation of cells was 1.4 μg/mg of protein, also accounting for 79% of the neutral glycolipid fraction. The purity of the isolated globoside, which migrated as two bands, was shown by HPTLC in two different solvent systems (Figure 2). It cochromatographed with authentic globoside and contained glucose, galactose, *N*-acetylgalactosamine, and long-chain base in a molar ratio of 1.00:2.11:0.98:0.92. The major fatty acids were C16:0 (9.5%), C18:0 (16.3%), C22:0 (23.3%), C24:1 (17.3%), and C24:0

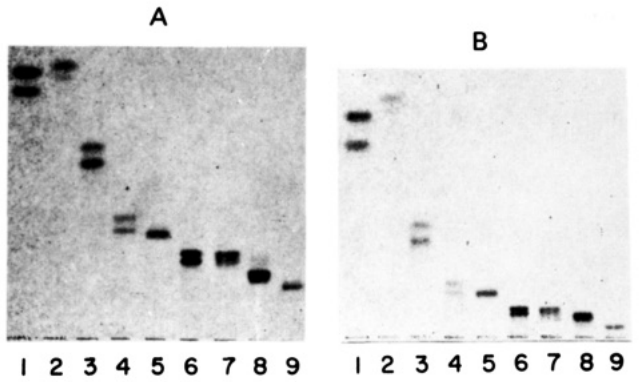


FIGURE 2: HPTLC of the isolated major neutral glycolipid from PC12 cells: (lane 1) GalCer from bovine brain; (lane 2) GlcCer from Gaucher's spleen (lanes 3, 4, and 6) LacCer, $Gb_3$, and $Gb_4$ from pig erythrocyte membranes, respectively; (lanes 5 and 9) asialo-$GM_2$ and asialo-$GM_1$ prepared from brain $GM_2$ and $GM_1$ gangliosides (Kasai et al., 1982); (lane 7) the isolated major neutral glycolipid from PC12 cell lines; (lane 8) $Gb_5$ from sheep erythrocyte membranes. Plate A was developed with solvent system A and plate B with solvent system B. The bands were visualized with α-naphthol/sulfuric acid reagent.

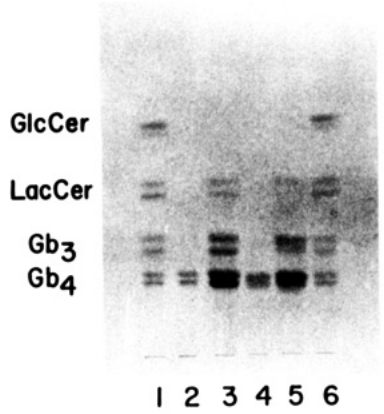


FIGURE 3: HPTLC of mild acid hydrolysis products of the major neutral glycolipid from PC12 cells: (lanes 1 and 6) GlcCer from Gaucher's spleen and LacCer, $Gb_3$, and $Gb_4$ from pig erythrocyte membranes; (lane 2) $Gb_4$; (lane 3) mild acid hydrolysis products of $Gb_4$ from pig erythrocyte membranes; (lane 4) the isolated major neutral glycolipid from PC12 cells; (lane 5) mild acid hydrolysis products of the sample in lane 4. The plate was developed with solvent system A, and the bands were visualized with α-naphthol/sulfuric acid reagent.

(23.6%). α-Hydroxy fatty acids accounted for less than 5% of the total fatty acids. The major long-chain base was C18 sphingenine (94.2%), with small amounts of C18 sphinganine (2.5%). No C20 homologues were detected.

Mild acid hydrolysis (0.1 N HCl, 30 min, 100 °C) of the major neutral glycolipid produced lactosylceramide, globotriaosylceramide, and a small amount of glucosylceramide (Figure 3). The negative ion FAB mass spectrum of the glycolipid showed prominent deprotonation peaks at $m/z$ 1337, 1335, 1309, 1281, 1253, and 1225, which were assigned to quasi-molecular ions of globoside with C24:0, C24:1, C22:0, C20:0, C18:0, and C16:0 fatty acids, respectively (Figure 4). These results clearly indicate that the major neutral glycolipid is likely to be globoside (globotetraosylceramide).

*Characterization of the Ganglioside Fraction.* The ganglioside fraction contained fucose, galactose, glucose, galactosamine, and sialic acid, which accounted for 17.4%, 25.7%, 10.9%, 17.7%, and 28.3% of the total carbohydrate constituents in this fraction, respectively. The major fatty acids were C16:0 (20.2%), C18:0 (15.6%), C20:0 (8.6%), C22:0 (10.5%), C24:0 (15.1%), and C24:1 (14.0%). The lipid-bound sialic acid concentration was 0.34 ± 0.03 (SEM, $N = 3$) and 0.18 ± 0.03 (SEM, $N = 3$) μg/mg of protein, for the NGF-treated and untreated cells, respectively. The difference is statistically significant ($p < 0.01$, Student's $t$ test for noncorrelated samples). After separation into mono-, di-, and polysialoganglioside fractions by DEAE-Sephadex column chromatography, HPTLC with a neutral solvent (C) showed three major bands in each fraction (Figure 5). The uppermost band in the monosialoganglioside fraction cochromatographed with authentic fucosyl-$G_{M1}$ (data not shown).

HPTLC–immunostaining of the PC12 cell gangliosides with anti-asialo-$G_{M1}$ antibody showed that all three fractions contained gangliosides with gangliotetraosyl backbones. The major band in the monosialoganglioside fraction, which was identified as fucosyl-$G_{M1}$, did not stain with anti-asialo-$G_{M1}$ antibody, nor did the other two bands, presumably because they also contain fucose (Margolis et al., 1984) (Figure 6, lanes 1 and 5). However, a small amount of $G_{M1}$ ganglioside, which was undetectable after spraying with the resorcinol–HCl reagent, was clearly demonstrated by the more sensitive immunostaining method. In the disialoganglioside fraction the immunostaining revealed the presence of $G_{D1a}$, $G_{T1a}$, and $G_{D1b}$ (Figure 6, lane 2). The two lower bands in the polysialoganglioside fraction, which constituted 67% of the total ganglioside by HPTLC–densitometry (Ando et al., 1978), are presumably $G_{T1b}$ and $G_{Q1b}$ (Figure 6, lane 3). This identification is consistent with previous findings by Margolis et al. (1983), who showed that these two bands could be converted to $G_{M1}$ following sialidase treatment, and with reports by

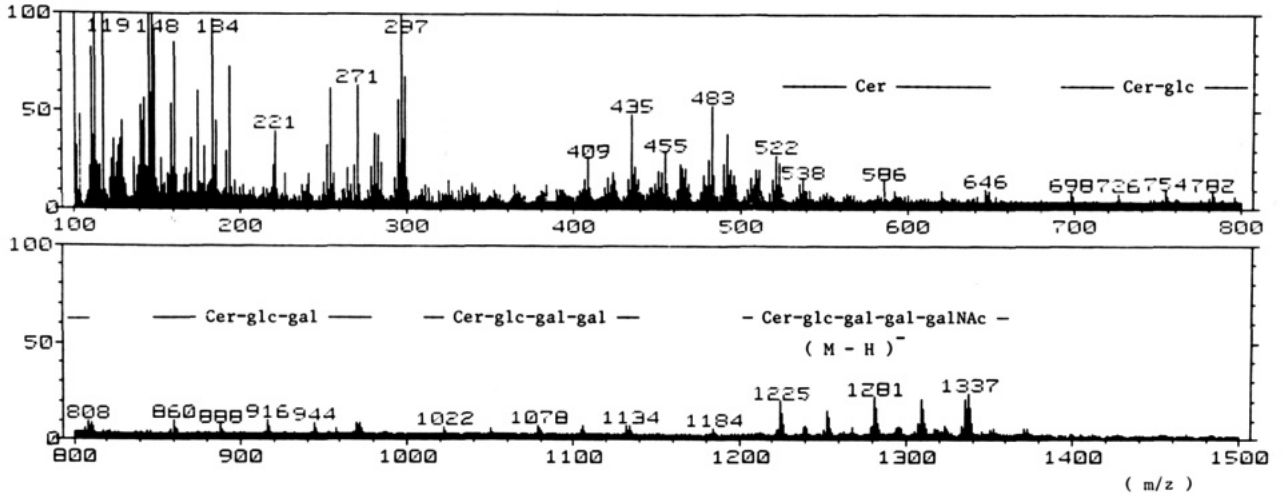


FIGURE 4: Negative ion FAB mass spectrum of the major glycolipid from PC12 cells.

Table II: Lipid Composition of PC12 Cells[a]

| | -NGF | | +NGF | |
|---|---|---|---|---|
| | HPTLC | LIT[b] | HPTLC | LIT[b] |
| cholesterol | 12.8 ± 0.2 | 0.261 ± 0.015[c] | 13.1 ± 0.4 | 0.248 ± 0.007[c] |
| cholesterol ester | 2.2 ± 0 | | 2.6 ± 0.1 | |
| sphingomyelin | 4.3 ± 0.1 | 6.7 ± 0.6 | 5.3 ± 0.1 | 5.7 ± 1.2 |
| choline phospholipids | 38.7 ± 0.6 | 40 ± 9.5 | 33.5 ± 1.1 | 45 ± 6.4 |
| ethanolamine phospholipids | 22.1 ± 0.6 | 24 ± 4.8 | 23.8 ± 0.4 | 23 ± 2.6 |
| inositol phospholipids | 7.2 ± 0.1 | 11.7 ± 3.7 | 7.3 ± 0 | 9.0 ± 2.6 |
| serine phospholipids | 6.4 ± 1.1 | 7.7 ± 4.4 | 8.4 ± 0.5 | 7.0 ± 1.0 |
| fatty acid | 6.3 ± 0.3 | ND | 6.1 ± 0.1 | ND |
| cardiolipin | ND[f] | 3.8 ± 0.7 | ND | 2.8 ± 0.7 |
| lysophosphatidylcholine | ND | 3.6 ± 4.0 | ND | 2.0 ± 0.4 |
| bis(monoacylglyceryl) phosphate | ND | 1.9 ± 1.1 | ND | 2.8 ± 2.7 |
| total lipid | 217.2 ± 2.1[d] | 178 ± 4.2[e] | 213.4 ± 2.5[d] | 165 ± 13[e] |

[a] All values are mean percent total ± SEM ($N = 3$). [b] Traynor et al. (1982). [c] Mole percent total cholesterol (moles of cholesterol per moles of cholesterol + moles of phospholipid). [d] Micrograms of lipid per milligram of protein. [e] Nanomoles lipid phosphorus per milligram of protein. [f] ND = not determined.

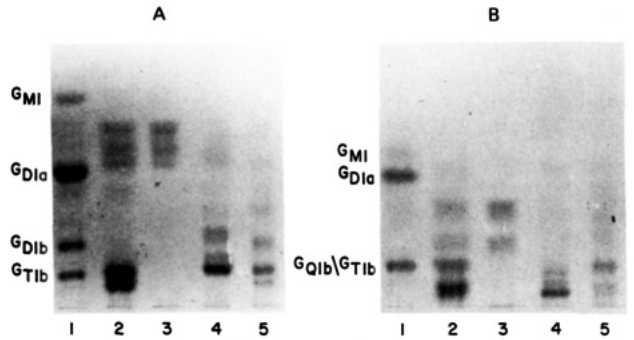


FIGURE 5: HPTLC of PC12 cell gangliosides: (lane 1) human brain ganglioside mixture; (lane 2) total gangliosides from PC12 cells; (lanes 3–5) mono-, di-, and polysialoganglioside fractions from PC12 cells, respectively. The plate was developed with solvent system C. The bands were visualized with resorcinol–HCl reagent.

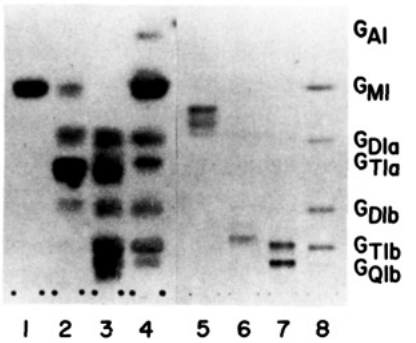


FIGURE 6: HPTLC–immunostaining of PC12 cell gangliosides: (lanes 1 and 5, 2 and 6, and 3 and 7) mono-, di-, and polysialoganglioside fractions separated by DEAE-Sephadex column chromatography, respectively; (lanes 4 and 8) human brain gangliosides. The plates were developed with solvent system C. The plate on the right was visualized with resorcinol–HCl reagent, and that on the left was stained with anti-asialo-$GM_1$ antibody after treatment with neuraminidase (Saito et al., 1985b).

Seifert (1981) and Dimpfel and Otten (1984). However, it should be emphasized that these assignments can only be regarded as tentative because it is possible that there are overlaps among some of the ganglioside species. In addition, since the amount of available material was low, further structural analysis could not be carried out. It should also be mentioned that the composition of individual gangliosides in NGF-treated cells did not differ significantly from that of untreated cells (unpublished observations).

*Non-Glycolipid Composition.* The lipid composition of PC12 cells grown in the presence or absence of NGF was determined by quantitative densitometric analysis with HPTLC. The results are shown in Table II, where they are compared with the data obtained by Traynor et al. (1982). The total lipid concentration of the NGF-treated cells was comparable to that of untreated cells, and the lipid composition did not change following NGF treatment. In both cases, the major lipids were cholesterol (13%), phosphatidylcholine (34–39%), and phosphatidylethanolamine (22–24%). The present findings are thus in agreement with those of Traynor et al. (1982).

## Discussion

One of the major findings of this study is that globoside constitutes the predominant neutral glycolipid of PC12 cells. This differs markedly from the neutral glycolipid composition of the human adrenal medulla, which contains 18% glucosylceramide, 23% galactosylceramide, 27% lactosylceramide, 20% globotriosylceramide, and only 12% globoside (Ariga et al., 1980). This also contrasts with brain, in which globoside is only a very minor constituent (3% of the neutral glycolipids and 0.07% of the total glycolipids in human cerebral cortex; Vanier et al., 1973). On the other hand, globoside has been reported to be present in the plasma membranes of sympathetic neurons cultured from newborn rats, and the amount of globoside detected by surface labeling of the neurons decreased by 60–70% when they were treated with heart cell conditioned medium (Zurn, 1982). This indicates that globoside levels in neurons (as well as in adrenal medullary cells) could be subject to a high degree of down regulation in vivo by factors that are not ordinarily present in culture medium. Also of possible relevance is the observation that a subpopulation (approximately 50%) of cultured newborn rat sensory neurons can be labeled with an antiserum directed against globoside, whereas cultured peripheral Schwann cells and central nervous system neurons and glia did not stain with this antiserum (Raff et al., 1979). This raises the possibility that the presence of significant amounts of globoside may be characteristic of developing peripheral nervous system cells to which PC12 cells may be compared.

An alternative to the possibility that the high globoside content in PC12 cells reflects their similarity to peripheral nervous system neurons is that this property relates to their transformed state. Alterations of glycolipid content and pattern have been reported for transformed cells in culture (Brady & Fishman, 1974) and have been suggested to be related to changes in surface properties such as decreased contact inhibition or exposure of tumor-specific antigens (Hakomori, 1981). In this regard, Laine and Hakomori (1973) reported that incorporation of exogenous globoside into

the plasma membranes of cultured hamster NIL cells resulted in changes in cell adhesiveness.

An additional point regarding PC12 cell globoside is that it contains normal fatty acids of variable chain length, but only traces of $\alpha$-hydroxy fatty acids. This fatty acid composition differs considerably from that of the erythrocyte membrane, which contains a large amount of the latter (Taketomi & Kawamura, 1972).

A second major aspect of our study is the finding that PC12 cell gangliosides contain a considerable proportion of ganglio-series polysialogangliosides as well as fucosyl-$GM_1$. Also, as reported previously (Margolis et al., 1983), NGF induced an increase in total ganglioside levels but caused no differential effects on the relative proportions of individual gangliosides (or other lipids). This contrasts with the morphological and biochemical alterations that NGF elicits in this cell line (Greene & Tischler, 1976, 1982). Thus, neurite outgrowth and neuronal differentiation, at least in the PC12 system, appear to neither require nor elicit extensive qualitative changes in lipid synthesis.

In a previous study of complex carbohydrates in PC12 cells (Margolis et al., 1983), it was concluded that they contained predominantly mono- and disialogangliosides that differed in certain respects from the corresponding brain gangliosides. This was based on the finding that all of the PC12 cell gangliosides could be eluted from DEAE-Sepharose CL-6B with concentrations of potassium acetate buffer that had been reported to elute mono- and disialogangliosides (Fredman et al., 1980) and the fact that the PC12 cell gangliosides did not exactly comigrate on HPTLC with any of the mono-, di-, or polysialogangliosides of brain.[2]

The thin-layer chromatographic pattern of resorcinol-stained PC12 cell gangliosides and their relationship to brain gangliosides (Figure 5) are essentially identical with those previously found for PC12 cell and brain gangliosides biosynthetically labeled from [$^{14}$C]- or [$^{3}$H]glucosamine (Margolis et al., 1983; Figure 1). The previous report of the presence of several fucosylmonosialogangliosides in PC12 cells (Margolis et al., 1984) is in agreement with the identification of fucosyl-$GM_1$ in the present study. Although Schwarting et al. (1986) identified two of these gangliosides as fucosyl-$G_{M2}$ and fucosyl-$G_{D2}$, their structures have never been fully established. In fact, a more recent study by Ariga et al. on gangliosides in PC12 cell transplanted tumor tissues suggested the presence of four different fucosylated gangliosides, which are shown to be fucosyl-$G_{M1}$, fucosyl-$G_{D1b}$, and the corresponding fucosylated gangliosides with blood group B determinant.[3]

The lack of exact HPTLC comigration of PC12 cell gangliosides with their counterparts from brain is probably due to the presence of fucose in most of the PC12 cell monosialogangliosides (Margolis et al., 1984) and to the different fatty acid and long chain base compositions of PC12 cell and brain gangliosides (which contain predominantly stearic acid and sphingenine and 4-eicosasphingenine bases; Ando & Yu, 1984), as revealed in the present study. It is well documented that differences in fatty acid and long chain base composition can contribute to different migratory rates of gangliosides on TLC (Ando & Yu, 1984).

The finding that PC12 cells contain high concentrations of ganglio-series gangliosides distinguishes them from adrenal medulla, which contains predominantly hematosides (Price et al., 1975; Ariga et al., 1980, 1982, 1983). The presence of high levels of brain and sympathetic neuron like polysialogangliosides in PC12 cultures may therefore reflect the neuronal rather than the chromaffin-like properties of these cells. On the other hand, the concentration of gangliosides in PC12 cells is considerably below that present in brain (Ledeen & Yu, 1982). The NGF-induced increase in ganglioside levels may thus reflect the neuronal differentiation that PC12 cells undergo in the presence of this factor.

[2] It was later learned that polysialogangliosides eluted in the "disialoganglioside" fraction, since in the published fractionation procedure that was used the buffer concentrations required to elute the different gangliosides were erroneously given for ammonium acetate rather than potassium acetate buffer (L. Svennerholm, personal communication).

[3] T. Ariga et al., unpublished data.

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**Registry No.** GlcCer, 85305-87-9; LacCer, 4682-48-8; $Gb_3$, 71965-57-6; $Gb_4$, 11034-93-8; $G_{M1}$, 104443-62-1; $G_{D1a}$, 104443-59-6; $G_{D1b}$, 104443-60-9; $G_{Q1b}$, 68652-37-9; $G_{T1a}$, 64522-98-1; $G_{T1b}$, 104443-58-5; NGF, 9061-61-4; fucosylganglioside $G_{M1}$, 71812-11-8; cholesterol, 57-88-5.

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# Chemistry of the Alkali-Labile Lesion Formed from Iron(II) Bleomycin and d(CGCTTTAAAGCG)†

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ABSTRACT: Two sets of products are formed from DNA upon treatment with Fe(II)·bleomycin + $O_2$. One set, which is believed to derive from a C-4′ hydroperoxy derivative of the DNA deoxyribose moiety, includes the four possible base propenals, as well as DNA oligomers having deoxynucleoside 3′-(phosphoro-2″-*O*-glycolates) at their 3′-termini. The other set of products consists of free bases and alkali-labile lesions, the latter of which had not previously been characterized structurally. By use of the self-complementary dodecanucleotide d(CGCTTTAAAGCG) having a site modified by Fe·bleomycin three nucleotides from the 5′-end, it has been possible to characterize the alkali-labile product as a C-4′ hydroxyapurinic acid. When the bleomycin-treated dodecanucleotide was treated with agents that effected decomposition of the alkali-labile lesion, products of the form CpGpx were obtained, and these proved useful for structural characterization of the alkali-labile lesion. Treatment with alkali produced CpGpx, where x was 2,4-dihydroxycyclopentenone. Alternatively, treatment with hydrazine provided a pyridazine derivative, and aqueous alkylamines led to formation of CpGp itself. The structures of all dinucleotides produced from the alkali-labile lesion were verified by direct comparison with authentic synthetic samples.

The bleomycins are antitumor antibiotics in use clinically for the treatment of certain neoplasms (Carter, 1978; Crooke, 1978; Umezawa, 1979). Bleomycin $A_2$ is the major constituent of the mixture of bleomycins employed in the clinic. The therapeutic effects of bleomycin are believed to be expressed at the level of DNA degradation; this process has been studied in cell-free systems and shown to proceed in the presence of any of several redox-active metal ions and $O_2$ (Ishida & Takahashi, 1975; Sausville et al., 1978; Ehrenfeld et al., 1984, 1985, 1987; Burger et al., 1984; Suzuki et al., 1985; Kuwahara et al., 1985) or *hν* (Sugiura et al., 1982; Chang & Meares, 1982, 1984).

The products of DNA-mediated strand scission have been shown to include base propenals (Burger et al., 1980; Giloni et al., 1981) and free bases (Burger et al., 1982; Wu et al., 1983), as well as oligonucleotides having the four possible nucleoside 3′-(phosphoro-2″-*O*-glycolates) at their 3′-termini (Giloni et al., 1981; Murugesan et al., 1985).

It has been known for some time that bleomycin also produces an alkali-labile lesion, subsequent base treatment of which results in DNA strand scission (Hecht, 1979). Structural elucidation of this lesion proved challenging, but was ultimately accomplished following base treatment of the alkali-labile lesion (Sugiyama, 1985c); more recently the proposed structure was verified following reduction of the lesion with $NaBH_4$ and subsequent digestion with $P_1$ nuclease and alkaline phosphatase (Rabow et al., 1986).

Presently, we describe fully the experiments that permitted identification (Sugiyama et al., 1985c) of the structures of the Fe·BLM[1]-derived alkali-labile lesion and its base-induced decomposition product. Also described are additional chemical transformations of the alkali-labile lesion that help to secure

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[1] Abbreviations: BLM, bleomycin; DMF, *N*,*N*-dimethylformamide; THF, tetrahydrofuran; HPLC, high-pressure liquid chromatography; DTT, dithiothreitol; ATP, adenosine 5′-triphosphate; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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