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# *O*-Acetylated Gangliosides in Bovine Buttermilk

## CHARACTERIZATION OF 7-*O*-ACETYL, 9-*O*-ACETYL, AND 7,9-DI-*O*-ACETYL $G_{D3}$*

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**Three *O*-acetylated gangliosides, $G_1$, $G_2$, and $G_3$, were purified from bovine buttermilk by using chloroform/methanol extraction, Folch partitioning, chromatography on DEAE-Sephadex A-25, and Iatrobeads columns. The final yields of gangliosides $G_1$, $G_2$, and $G_3$ were 2 mg, 37 mg, and 40 mg per 1.7 kg of the buttermilk powder, respectively. On the basis of immunostaining on high performance thin layer chromatography with specific monoclonal antibodies, mild alkaline treatment, gas-liquid chromatographic analysis, fast atom bombardment mass spectrometry, and proton nuclear magnetic resonance studies, $G_1$ and $G_2$ are characterized as *O*-acetylated $G_{D3}$ and $G_3$ as *O*-acetylated $G_{T3}$, and the structures of these gangliosides are as follows:**

**$G_1$: NeuAc(α2→8)NeuAc(α2→3)Gal(β1→4)-**
**|  Glc(β1→1′)Ceramide**
**(7-,9-)**
**|**
***O*-$Ac_2$**

**$G_2$: NeuAc(α2→8)NeuAc(α2→3)Gal(β1→4)-**
**|  Glc(β1→1′)Ceramide**
**(7-) or (9-)**
**|**
***O*-Ac**

**$G_3$: NeuAc(α2→8)NeuAc(α2→8)NeuAc(α2→3)-**
**|  Gal(β1→4)Glc(β1→1′)Ceramide**
**|**
***O*-Ac**

**The major fatty acids of these gangliosides were C18:0, C22:0, C23:0, and C24:0, and the long chain base was C18-sphingosine.**

The sialic acids are a diverse family of 9-carbon sugars found as terminal monosaccharides on many glycoconjugates (3, 4). During the last few years, there has been a growing interest in the occurrence, metabolism, and function of *O*-acetylated sialic acids of glycoproteins and gangliosides. *O*-Acetyl esters at the 9-position of sialic acids show tissue-specific expression, developmental regulation, and regional distribution in a variety of tissues and sometimes reappear as oncofetal antigens (5–14). The 9-*O*-acetylated disialoganglioside (9-*O*-acetyl $G_{D3}$) is selectively expressed in the embryonic adrenal gland, the mesonephron, and certain regions of the developing nervous system, but not in other parts of the fetus (13–16). Studies with the monoclonal antibody JONES have demonstrated that this unusual ganglioside shows a dorsal-ventral gradient across the developing retina, a distribution distinct from its precursor, $G_{D3}$ (6, 8, 17). Similar discordance between $G_{D3}$ and its *O*-acetylated counterpart is found in other areas of the developing central and peripheral nervous system (8–10, 17–19). Expression of 9-*O*-acetyl $G_{D3}$ decreases shortly after birth and in the adult animal appears confined to the adrenal medulla, the renal glomerular polocyte, and a few cells in the nervous system (8–10, 17, 18). It was found that only the retina and the adrenal gland showed variable abnormalities in organization, when *O*-acetyl groups in 9-*O*-acetylated gangliosides were selectively destroyed during embryogenesis by expressing the 9-*O*-acetylsialic acid-specific esterase of influenza C (19). These observations suggest that 9-*O*-acetylated sialic acids may play an important role in murine development at the 2-cell stage and in certain differentiated tissues.

We previously reported the ganglioside composition of hamster melanomas, in which $G_{M3}$, $G_{D3}$, and *O*-acetyl $G_{D3}$ were expressed as the major gangliosides (20). The expression of *O*-acetyl $G_{D3}$ in melanoma cells suggests that its appearance may serve to modulate and provide selectivity for the critical events of cell metastasis and growth in melanoma cells (20). In spite of such intriguing clues, little is known about the structures and the specific biological roles of these *O*-acetylated gangliosides during development. The acetyl group in the *O*-acetylated $G_{D3}$ appears to be localized at the 9-position of the terminal sialic acid moiety (14, 16, 22–24). It is important, therefore, to find a good source for the large scale purification of these gangliosides. Recently, we found in bovine buttermilk an *O*-acetyl $G_{D3}$ species (21) which, based on its reactivity with specific monoclonal antibodies, seemed to be the same as those expressed in developing nervous system as well as melanoma tissues (13). A more detailed study revealed that there were several different species of *O*-acetylated gangliosides whose structures had not yet been deduced. In the present paper, we describe the purification and char-

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acterization of three *O*-acetylated gangliosides from bovine buttermilk and their reactivity toward the monoclonal antibodies, D1.1 (5, 11), 18B8 (29), and A2B5 (30).

## EXPERIMENTAL PROCEDURES

*Materials*—Bovine buttermilk powder was from Land O'Lakes (Minneapolis, MN). High performance TLC plates (nanoplates, 10 × 20 cm) were purchased from E. Merck, Darmstadt, Federal Republic of Germany. DEAE-Sephadex A-25 and Iatrobeads were from Pharmacia Fine Chemicals, Uppsala, Sweden, and Iatron Laboratories, Inc., Tokyo, Japan, respectively. The monoclonal antibody R24 was a generous gift from Dr. Kenneth Lloyd (Memorial Sloan-Kettering Cancer Center, New York, NY), the monoclonal antibody D1.1 was from Dr. Joel Levine (SUNY, Stony Brook, NY), and the monoclonal antibodies A2B5 and 18B8 were from Dr. George Grunwald (Jefferson Medical College, Philadelphia, PA). $^{125}$I-Labeled staphylococcal protein A was supplied by Du Pont-New England Nuclear. Horseradish peroxidase-conjugated goat anti-mouse IgM or IgG was from Cappel, West Chester, PA. Anhydrous pyridine, hexamethyldisilazane, and trimethylchlorosilane were purchased from Pierce Chemical Co. All other chemicals were of HPLC[1] or analytical grade.

*Purification of Total Gangliosides*—Bovine buttermilk powder, 1.7 kg, containing 32% protein, 8.5% minerals, 49% carbohydrates, and 3.5% other small molecular weight compounds, was suspended in water and then dialyzed against distilled water to remove small oligosaccharides, minerals, and other small moleculer weight material. After lyophilization, 1 ml of 0.9% NaCl per g of dry dialyzed buttermilk powder was added to facilitate the ensuing extraction. The total lipids were extracted from the lyophilized materials with 10 volumes each of chloroform:methanol (C:M) (2:1, 1:1, and 1:2 v/v) (25). The combined lipid extracts were adjusted to a ratio of C:M:water (W) (2:1:0.6) and then partitioned three times (26). The ganglioside fractions from the upper phases were combined, evaporated, and dialyzed against deionized water for 3 days with frequent changing of water and then lyophilized. The residue containing gangliosides was dissolved in C:M:W (30:60:8) (solvent A) and applied onto a DEAE-Sephadex A-25 chromatographic column (100 × 2.0 cm, inside diameter). The column was washed with 10 volumes of solvent A to remove neutral lipids, then the crude gangliosides were eluted stepwise with a solvent system of 1,000 ml each of 0.05 M, 0.2 M, and 0.8 M sodium acetate in solvent A. One hundred eighty 15-ml effluent fractions were collected. On the basis of the elution profile, fractions 1–80 were pooled as monosialogangliosides, 80–110 as disialogangliosides, and 110–140 as trisialogangliosides. The solvent in each fraction was evaporated. After removing the sodium acetate in each fraction by dialysis, individual gangliosides in the di- and trisialoganglioside fractions were separated on a Iatrobeads column (100 × 2.0 cm) with gradient elution using 1 liter each of C:M:0.5% $CaCl_2 \cdot 2H_2O$ (70:30:3 and 35:65:5, v/v for disialogangliosides) or C:M:0.5% $CaCl_2 \cdot 2H_2O$ (60:40:5 and 35:65:10, v/v for trisialogangliosides).

Final purification of individual gangliosides was achieved by Iatrobeads column (110 × 0.4 cm) chromatography as follows. *O*-Acetylated $G_{D3}$ was purified by gradient elution using C:M:W (70:30:3 and 35:65:5, v/v) and *O*-acetylated $G_{T3}$ by gradient elution using C:M:W (65:35:5 and 30:70:10, v/v). The purity of each *O*-acetylated ganglioside species was examined by high performance thin layer chromatography (HPTLC) with two different solvent systems: (*a*) *n*-propyl alcohol:water (80:20, v/v) and (*b*) C:M:0.5% aqueous $CaCl_2 \cdot 2H_2O$ (55:45:10, v/v). The gangliosides were visualized on the plate by spraying with the resorcinol-HCl reagent (27) followed by heating at 95 °C for 30 min.

*Mild Alkali Treatment of O-Acetylated Gangliosides*—One hundred μg of a ganglioside sample was treated with 0.2 N methanolic NaOH at 37 °C for 2 h. The reaction mixture was then neutralized with 0.2 N HCl, and the salts in the mixture were removed by a Sephadex LH-20 column (47 × 0.5 cm, inside diameter; 23-ml bed volume) by elution with C:M:W (30:60:8).

*HPTLC Overlay of O-Acetylated Gangliosides*—HPTLC overlay was carried out according to Kasai *et al.* (28). Briefly, the gangliosides were chromatographed on an HPTLC plate with the developing solvent system of C:M:0.5% aqueous $CaCl_2 \cdot 2H_2O$ (55:45:10, v/v). After chromatography, the plate was dried *in vacuo* for 2 h and dipped in a solution of 0.4% poly(isobutyl methacrylate) in *n*-hexane for 1 min and dried *in vacuo*. The plate was incubated with 1% BSA in PBS (BSA/PBS) (pH 7.4) at room temperature for 90 min and then overlaid with monoclonal antibodies D1.1, A2B5, or 18B8 at a dilution of 1:50–100 in BSA/PBS. After incubation at room temperature for 90 min, the plate was washed with PBS five times. The plate overlaid with D1.1 was covered with horseradish peroxidase-conjugated goat anti-mouse IgM or IgG at a dilution of 1:100 in BSA/PBS for 90 min and washed as described above. After washing, the plate was incubated for a few minutes with 0.5 mg/ml 4-chloro-1-naphthol and 0.01% $H_2O_2$ as substrate.

The plates overlaid with A2B5 and 18B8 were covered by goat anti-mouse Ig at a dilution of 1:100 in BSA/PBS for 60 min. After washing with PBS, the plates were covered with 2 ml of $^{125}$I-labeled staphylococcal protein A ($10^6$ cpm/ml), incubated for 60 min, and washed with 0.1% Triton X-100 in 0.9% NaCl, followed by exposure to an x-ray film.

*Compositional Analysis*—To determine the sugar and fatty acid compositions, gas-liquid chromatographic (GLC) analysis was carried out according to the procedure of Ariga *et al.* (31) with slight modifications. A ganglioside sample, containing about 50 μg of sialic acid, was subjected to methanolysis for 18 h at 75 °C with 1 ml of 1% methanolic hydrogen chloride. Following methanolysis, the resulting fatty acid methyl esters were extracted with *n*-hexane, and the extract was evaporated and dissolved in 50 μl of *n*-hexane. An aliquot of this solution was injected onto a column of HP5 (cross-linked 5% PhMe Silicone, 25 m × 0.2 mm × 0.5 μm film thickness) maintained at 270 °C for analysis of fatty acids. The lower methanolic layer was evaporated under a stream of nitrogen and dried *in vacuo*. The dried residue was treated with 100 μl of hexamethyldisilazane: trimethylchlorosilane:pyridine (1.3:0.8:1,v/v) at 60 °C for 5 min, and the resulting trimethylsilylated derivatives of the saccharides were analyzed by GLC on the HP5 column programmed at 3 °C/min from 220 to 270 °C.

*Fast-atom Bombardment (FAB) Mass Spectrometry*—Negative ion FAB mass spectra were obtained by a TSQ 70 triple-stage quadrupole mass spectrometer (Finnegan MAT Inc., San Jose, CA) equipped with a FAB ion source. Xenon gas was used at 8 kV as an ionization beam. A ganglioside sample, 30 μg, was dissolved in 10 μl of C:M (1:1, v/v). Triethanolamine:tetramethylurea (1:1, v/v, 2 μl) was added, and the solvent mixture (about 1 μl) was applied to a stainless steel sample holder. The sample was analyzed as described previously (31).

*Proton Nuclear Magnetic Resonance Spectroscopy*—Samples were prepared for $^1$H NMR analysis as described previously (32, 33). Briefly, each sample, 2 mg, was dissolved in 0.5 ml of $D_2O$ and lyophilized to remove exchangeable protons. The residue was dissolved in 0.5 ml of dimethyl sulfoxide-$d_6$/$D_2O$ (98:2, v/v). NMR spectra were obtained on spectrometers operating at $^1$H frequencies of 300 MHz or 500 MHz.

Resonance assignments were made largely on the basis of a 300-MHz two-dimensional Homonuclear Hartman-Hahn spectroscopy (HOHAHA) data set which was acquired with a mixing time of 80 ms and a spin lock field strength of 8 kHz. This data set consisted of 384 $t_1$ increments. Each $t_1$ increment consisted of 1024 complex points over a 2401-Hz sweep width averaged for 256 transients with a 1.1 second repetition rate. Quadrature detection was achieved in the $f_1$ dimension using hypercomplex phase cycling with real and imaginary $t_1$ points stored separately. Data were processed for phase-sensitive display using FELIX 2.0 (Hare Research, Bothell, WA) with cosine bell weighing functions and zero filling in both dimensions to yield a 2K × 2K data matrix.

## RESULTS

After Folch partitioning, 4.12 g of lipid extract were recovered from 0.67 kg of lyophilized materials after dialysis. About 360 mg of mono-, 680 mg of di-, and 196 mg of trisialoganglioside fractions were recovered from the DEAE-Sephadex A-25 column (see "Experimental Procedures"). Fig. 1 shows the elution profile for bovine buttermilk gangliosides. At least three alkali-labile gangliosides, which were designated as $G_1$, $G_2$, and $G_3$, could be detected on the basis of mild alkali

[1] The abbreviations used are: HPLC, high performance liquid chromatography; HPTLC, high performances thin layer chromatography; $^1$H NMR, proton-nuclear magnetic resonance; HOHAHA, homonuclear Hartman-Hahn spectroscopy; FAB, fast-atom bombardment; BSA/PBS, bovine serum albumin in phosphate-buffered saline; mAb, monoclonal antibody. The ganglioside nomenclature follows the system of Svennerholm (1) and a recent IUPAC-IUB recommendation (2).

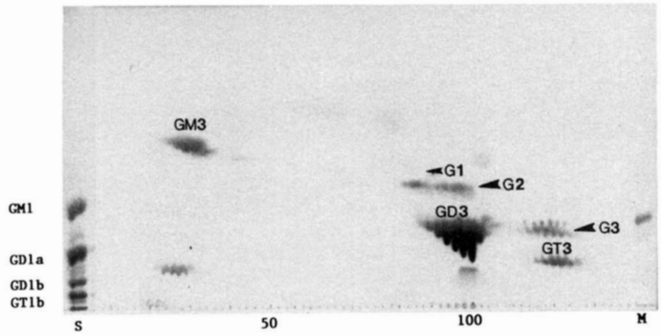


FIG. 1. **HPTLC ganglioside profile of bovine buttermilk.** The profile was obtained by eluting the gangliosides from a DEAE-Sephadex A-25 column. Two $\mu$l from each of individual fractions were spotted on the plate. The developing solution was solvent system b (see "Experimental Procedures"). The spots were located with resorcinol-HCl reagent. *Lane S*, standard gangliosides; *lane M*, a mixture of gangliosides extracted from bovine buttermilk. Three acetylated gangliosides, designated as $G_1$, $G_2$, and $G_3$, were recognized on the profile.

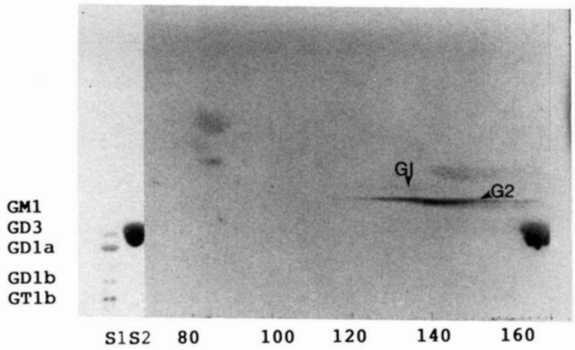


FIG. 2. **HPTLC purification profiles of *O*-acetylated $G_{D3}$ gangliosides from bovine buttermilk.** *O*-Acetylated $G_{D3}$ was purified from the disialoganglioside fraction by Iatrobeads column chromatography. The developing solution was solvent system b (see "Experimental Procedures"). The spots were located with resorcinol-HCl reagent. *Lane S1*, standard gangliosides; *lane S2*, standard ganglioside $G_{D3}$. *O*-Acetylated ganglioside $G_{D3}$ was separated from $G_{D3}$ and could be recognized on the plate.

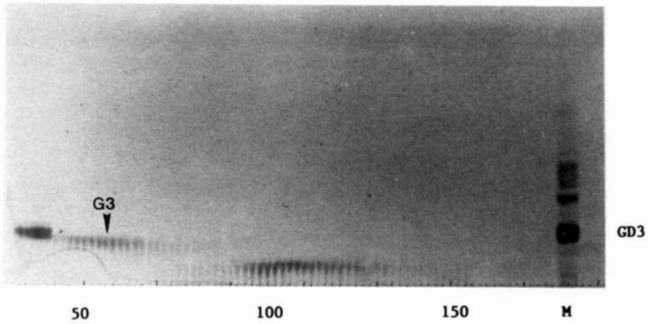


FIG. 3. **HPTLC purification profiles of *O*-acetylated $G_{T3}$ gangliosides from bovine buttermilk.** *O*-Acetylated $G_{T3}$ was separated from trisialoganglioside $G_{T3}$, which is around fraction 100, by Iatrobeads column chromatography. Other conditions were the same as described in Fig. 2.

treatment as described later. As previously reported (34), bovine buttermilk was found to contain 0.92 $\mu$mol of lipid-bound sialic acid/g dry weight. The concentrations of acetylated gangliosides $G_1$, $G_2$, and $G_3$ are 0.4% (2 mg), 5% (37 mg), and 6% (40 mg) of the total lipid-bound sialic acid, respectively. Figs. 2 and 3 show the ganglioside elution profiles from Iatrobeads column chromatographies. Three gangliosides, $G_1$ to $G_3$, were isolated and purified to homogeneity. Ganglioside $G_1$ migrated slightly further than ganglioside $G_2$, while $G_2$ migrated between $G_{M2}$ and $G_{M1}$, and $G_3$ between $G_{D3}$ and $G_{D1a}$. Table I shows the results of compositional analysis of the carbohydrates and fatty acids of $G_{D3}$, $G_1$, $G_2$, $G_{T3}$, and $G_3$. Gangliosides $G_1$ and $G_2$ contain glucose, galactose, and sialic acid in a molar ratio of 1:1:2, and $G_3$, 1:1:3, respectively. The fatty acid composition of these gangliosides is shown in Table I. The major fatty acids were stearic, behenic, tricosanoic, and tetracosanoic acids.

The purified gangliosides, $G_1$ to $G_3$, were subjected to alkaline treatment (see "Experimental Procedures"). After mild alkaline treatment, the degradation products from gangliosides $G_1$ and $G_2$ co-migrated on TLC with authentic $G_{D3}$, while $G_3$ co-migrated with $G_{T3}$, as shown in Fig. 4. These data suggest that these alkali-labile gangliosides may contain *O*-acetylated sialic acid residues.

Fig. 5 shows the negative ion-FAB mass spectra and fragmentation diagrams of gangliosides $G_{D3}$, $G_1$, and $G_2$, which were isolated from bovine buttermilk. In the mass spectra of $G_{D3}$ (Fig. 5*A*), $G_1$ (Fig. 5*B*), and $G_3$ (Fig. 5*C*), five ion groups are clearly discernible which arise from ceramide and ceramide-bearing fragment ions. In the mass spectrum of $G_{D3}$, the prominent molecular ions $[M + Na - 2H]^-$ are clearly seen

TABLE I

*Sugar and fatty acid compositions of gangliosides isolated from bovine buttermilk*

| | Gangliosides | | | | |
|---|---|---|---|---|---|
| | $G_{D3}$ | $G_1$ | $G_2$ | $G_3$ | $G_{T3}$ |
| Sugars | | | | | |
| Glc | 1 | 1 | 1 | 1 | 1 |
| Gal | 1.09 | 0.98 | 0.85 | 1.02 | 1.01 |
| Sialic acid | 2.16 | 1.79 | 1.85 | 2.98 | 2.87 |
| Fatty acid (%) | | | | | |
| 14:0 | 2 | Trace | Trace | Trace | 2.5 |
| 16:1 | 0 | 0 | Trace | Trace | 0 |
| 16:0 | 7.7 | 1.9 | 2.2 | 10.8 | 7.4 |
| 18:1 | Trace | 3.3 | 11.8 | 6.6 | 10.7 |
| 18:0 | 3.7 | 7.4 | 22.4 | 38.3 | 15.6 |
| 20:1 | Trace | Trace | Trace | Trace | Trace |
| 20:0 | 1.6 | 1.2 | 1.0 | 2.6 | 1.4 |
| 21:0 | 1.7 | Trace | 1.2 | Trace | 1.2 |
| 22:0 | 27.3 | 25.0 | 15.7 | 18.0 | 19.1 |
| 23:0 | 31.7 | 30.9 | 19.6 | 13.0 | 21.4 |
| 24:1 | 2.9 | 2.5 | 2.1 | Trace | 1.5 |
| 24:0 | 19.3 | 25.2 | 18.6 | 6.7 | 19.1 |
| 25:0 | 1.5 | 1.4 | 1 | Trace | Trace |

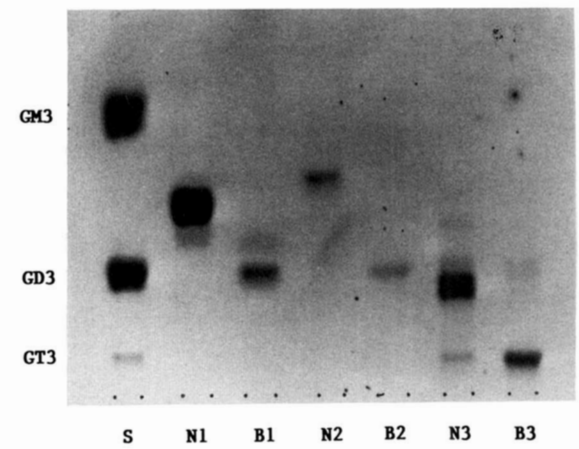


FIG. 4. **HPTLC of purified *O*-acetylated gangliosides treated with methanolic NaOH.** *Lane 1*, standard gangliosides; *lanes N1, N2,* and *N3* correspond to untreated $G_2$, $G_1$, and $G_3$, respectively; *lanes B1, B2,* and *B3* to treated $G_2$, $G_1$, and $G_3$, respectively. Other conditions were the same as described in Fig. 3. The bands were visualized by spraying with the resorcinol-HCl reagent.

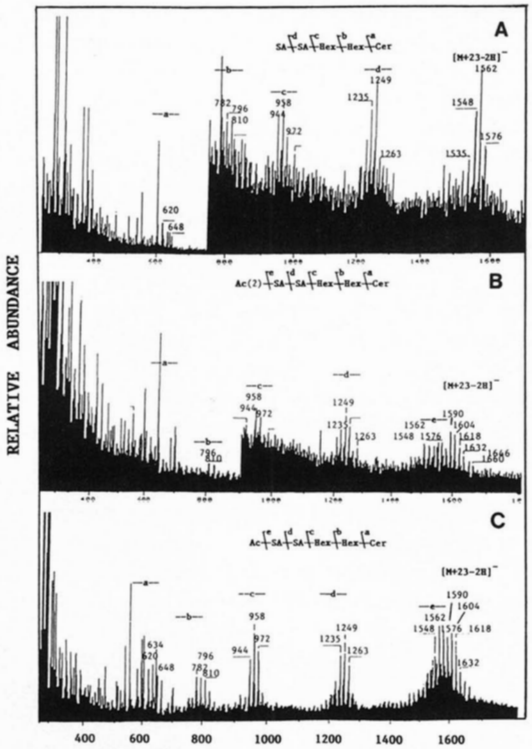


FIG. 5. **The negative ion-FAB mass spectra and fragmentation diagrams of $G_{D3}$ (*A*), $G_1$ (*B*), and $G_2$ (*C*).**

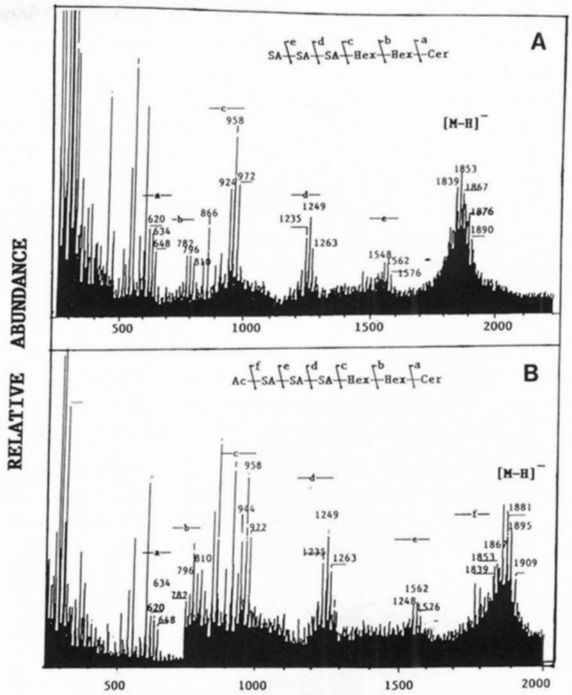


FIG. 6. **The negative ion-FAB mass spectra and fragmentation diagrams of $G_{T3}$ (*A*) and $G_3$ (*B*).**

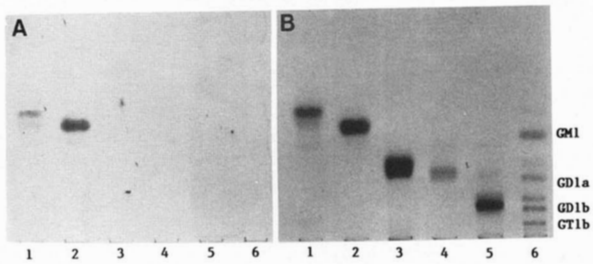


FIG. 7. **Detection of *O*-acetylated ganglioside $G_{D3}$ on HPTLC plate by immunoreactivity with monoclonal antibody D1.1.** *A*, gangliosides reacting with monoclonal antibody D1.1 and horseradish peroxidase conjugate (see "Experimental Procedures"; *B*, gangliosides visualized with the resorcinol-HCl reagent. *Lanes 1–5* correspond to $G_1$, $G_2$, $G_{D3}$, $G_3$, and $G_{T3}$, respectively; *lane 6*, standard gangliosides. Each lane contains 2–5 $\mu$g of lipid-bound sialic acid.

in which the major ions at *m/z* 1548, 1562, and 1576 provide information on the molecular weights of $G_{D3}$ molecular species with C18-sphingosine and C22:0, C23:0, and C24:0 fatty acids, respectively. These ions are also present in the spectra of $G_1$ and $G_2$. However, the quasimolecular ions for $G_1$ and $G_2$ are shifted by 84 and 42 mass units from those observed for $G_{D3}$, respectively, suggesting the presence of two and one *O*-acetyl groups in $G_1$ and $G_2$, respectively. For example, the ions at *m/z* 1632, 1646, and 1660 in $G_1$ correspond to di-*O*-acetyl $G_{D3}$ ganglioside with C18-sphingosine and C22:0, C23:0, and C24:0 fatty acids, respectively. In the case of $G_2$, these ions are of relatively low intensity, but the ions 1590, 1604, and 1618 are prominent, which provides information on the molecular weight of the monoacetyl $G_{D3}$ molecular species.

In all spectra in Fig. 5, *A*, *B*, and *C*, the fragment ions corresponding to the formal elimination of 1 or 2 sialic acid units and 2 hexose units from the molecular ions are detected at *m/z* 1235, 1249, and 1263 (fragment group d), *m/z* 944, 958, and 972 (fragment group c), *m/z* 782, 796, and 810 (fragment group b), and *m/z* 620, 634, and 648 (fragment group a), respectively. Owing to the detection of three ions, *m/z* 1235, 1249, and 1263 in the spectra of both $G_1$ and $G_2$ (Figs. 5, *B* and *C*), the *O*-acetyl groups must be attached to the terminal sialic acid in the molecule.

Fig. 6 shows the FAB mass spectra of $G_{T3}$ and $G_3$. In both spectra, six major ion groups are present, which arise from ceramide and ceramide-bearing fragment ions. In both spectra, ion regions are complex, probably due to the presence of quasimolecular ions containing $[M - H]^-$ and $[M + Na - 2H]^-$. For example, prominent molecular ions $[M - H]^-$ are detected at *m/z* 1839, 1853, and 1867 which correspond to the molecular species with C18-sphingosine and C22:0, C23:0, and C24:0 fatty acid, respectively. The less prominent ions at *m/z* 1876 and 1890 correspond to $[M + Na - 2H]^-$ ions with C18-sphingosine and C23:0 and C24:0 fatty acids, respectively. In the spectra of $G_3$, all these ions are shifted by 42 mass units; therefore, $G_3$ is supposed to be mono-*O*-acetyl $G_{T3}$. In both spectra, three major fragment ions corresponding to the successive elimination of NeuAc (*e*), NeuAc (*d*), NeuAc (*c*), hexose (Gal) (*b*), and hexose (Glc) (*a*) are detected at *m/z* 1548, 1562, and 1576 for $G_{D3}$, *m/z* 1235, 1249, and 1263 for $G_{M3}$, *m/z* 944, 958, and 972 for LacCer, *m/z* 782, 796, and 810 for GlcCer, and *m/z* 620, 634, and 648 for ceramide, respectively. Since the ions *m/z* 1548, 1562, and 1576 corresponding to $G_{D3}$ are not shifted, the *O*-acetyl group of $G_{T3}$ must also be attached to the terminal sialic acid residue in the molecules.

In order to confirm the structures of the alkali-labile gangliosides, gangliosides $G_1$, $G_2$, and $G_3$ were analyzed by the HPTLC-overlay method using specific monoclonal antibodies against *O*-acetylated gangliosides. As shown in Figs. 7 and 8, $G_1$ and $G_2$ reacted with the monoclonal antibody D1.1 which is specific for 9-*O*-acetyl $G_{D3}$ (14), and $G_3$ reacted with the monoclonal antibody A2B5 (29) but did not react with 18B8 which is specific for $G_{T3}$ (30). The product of $G_3$ after mild alkaline treatment, however, reacted with 18B8. These data suggest that gangliosides $G_1$ and $G_2$ contain *O*-acetylated $G_{D3}$ and $G_3$ is *O*-acetylated $G_{T3}$.

Although the mass spectrometric and immunochemical data provide clear evidence for the presence of both mono-

and di-*O*-acetylated gangliosides, these data cannot provide us with any information concerning the sites of *O*-acetyl substitution on the terminal α-NeuAc residue. Such data can be provided by nuclear magnetic resonance assignment data for these compounds as the chemical shifts of resonances corresponding to the sites of *O*-acetylation are likely to differ significantly from the same protons in the unsubstituted gangliosides. These resonance assignments, which were made on the basis of a two-dimensional HOHAHA data set, are summarized in Table II. In addition, the regions of the HOHAHA data set which contain the downfield shifted resonances corresponding to the sites of *O*-acetylation are provided in Fig. 9.

From the data in Table II and in Fig. 9, it is clear that the sample used for NMR analysis consisted of a mixture of mono- and disubstituted $G_{D3}$. In Fig. 9*A*, we present the region of the HOHAHA data set containing the downfield shifted resonances from the monosubstituted species ($G_2$). From these data, it is clear that there are two distinct monosubstituted species. In one species, the most striking feature was the resonance at 4.82 ppm. This resonance was assigned to the H9b proton which is shifted downfield by more than 1 ppm from the H9b resonance position in unsubstituted $G_{D3}$. In addition, significant downfield shifts were noted for the H9a and H8 resonances. Based on these chemical shift differences, we conclude that this monosubstituted ganglioside corresponds to 9-*O*-Ac $G_{D3}$. In the other monosubstituted ganglioside, the most significant downfield shift is observed for the H7 resonance which is shifted to 4.15 ppm from its position at 3.21 ppm in unsubstituted $G_{D3}$. In addition, we noted a significant downfield shift for the H8 resonance in this ganglioside. On the basis of these shift differences, we conclude that this ganglioside corresponds to 7-*O*-Ac $G_{D3}$. Although shift perturbations were also noted for the H9a and H9b resonances in 7-*O*-Ac $G_{D3}$, these perturbations were somewhat smaller in magnitude than the perturbations noted for downfield shifts noted for the H9a and H9b resonances in 9-*O*-Ac $G_{D3}$. These data suggest that $G_2$ ganglioside is a mixture of 7-*O*-acetyl and 9-*O*-acetyl $G_{D3}$.

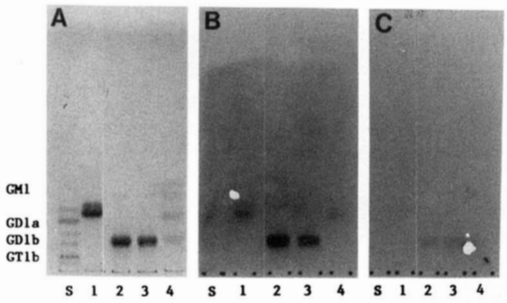


FIG. 8. **Detection of *O*-acetylated ganglioside $G_{T3}$ on HPTLC plate by immunoreactivity with the monoclonal antibodies A2B5 and 18B8 (see "Experimental Procedures").** *A*, the bands were visualized by spraying with the resorcinol reagent; *B*, gangliosides reacting with the monoclonal antibody A2B5; *C*, gangliosides reacting with the monoclonal antibody 18B8. *Lanes 1, 2, 3,* and *4* correspond to *O*-Ac $G_{T3}$ ($G_3$), base-treated *O*-Ac $G_{T3}$ ($G_{T3}$), $GT_3$, and a mixture of trisialogangliosides from bovine buttermilk, respectively; *lane S*, standard gangliosides. Each lane contains 2 μg of lipid-bound sialic acid.

In Fig. 9*B*, we present the region of the two-dimensional HOHAHA data set containing the downfield shifted resonances for disubstituted species ($G_1$). Comparison of the data in Fig. 9*B* with the data shown in Fig. 9*A* shows that the spectrum of the di-*O*-acetyl species is similar to the spectrum which would be obtained by summing the spectra of the two mono-substituted $G_{D3}$ species in Fig. 9*A*. These data are largely confirmed in the assignment data presented in Table II. In this ganglioside ($G_1$), we see large downfield shifts for the H7, H8, H9a, and H9b resonances. Therefore, we conclude that this diacetylated species corresponds to (7, 9)-di-*O*-Ac $G_{D3}$. Closer examination of the data in Table II indicates that the resonance positions for the H9a and H9b resonances are not identical with the positions observed in 9-*O*-Ac $G_{D3}$. These differences are likely indicative of some interaction between the two *O*-acetyl groups in the disubstituted species. Combined with the data from mobilities on HPTLC, mass spectrometric, and TLC immunostaining analysis, the results suggest a structure of $G_1$ most consistent with 7,9-di-*O*-acetyl $G_{D3}$ (acetyl$_2$-*O*-7, 9NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1→

TABLE II

*Chemical shifts for gangliosides $G_{D3}$ and O-acetyl $G_{D3}$*

| | Residue | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | H-1 | H-2 | H-3 | H-4 | H-5 | H-6 | H-7 | H-8 | H-9 |
| $G_{D3}$[a] | | | | | | | | | |
| I. | 4.16 | 3.05 | 3.31 | 3.28 | 3.28 | 3.60(a)<br>3.73(b) | | | |
| II. | 4.27 | 3.28 | 3.63 | 3.87 | 3.35 | 3.45(a)<br>3.47(b) | | | |
| A. | | | 1.69(a)<br>2.34(e) | 3.83 | 3.57 | 3.14 | 3.43 | 3.43 | 3.55(a)<br>3.81(b) |
| C. | | | 1.39(a)<br>2.77(e) | 3.46 | 3.57 | 3.33 | 3.21 | 3.48 | 3.32(a)<br>3.58(b) |
| *O*-Ac $G_{D3}$ | | | | | | | | | |
| I. | 4.12 | 3.02 | 3.30 | 3.24 | 3.43 | 3.63(a)<br>3.71(b) | | | |
| II. | 4.30 | 3.30 | 3.83 | | | | | | |
| A. | | | 1.47(a)<br>2.35(e) | 3.77 | 3.49 | 3.15 | 3.42 | 3.42 | 3.50(a)<br>3.80(b) |
| C.(7-*O*-) | | | 1.40(a)<br>2.70(e) | 3.49 | 3.39 | 3.28 | 4.15 | 3.92 | 3.15(a)<br>3.75(b) |
| (9-*O*-) | | | 1.40(a)<br>2.70(e) | 3.49 | 3.39 | 3.28 | 3.27 | 3.82 | 3.90(a)<br>4.82(b) |
| (7,9-*O*-) | | | 1.40(a)<br>2.70(e) | 3.49 | 3.39 | 3.28 | 4.26 | 4.06 | 3.69(a)<br>5.02(b) |

[a] Cited from Refs. 32 and 33.

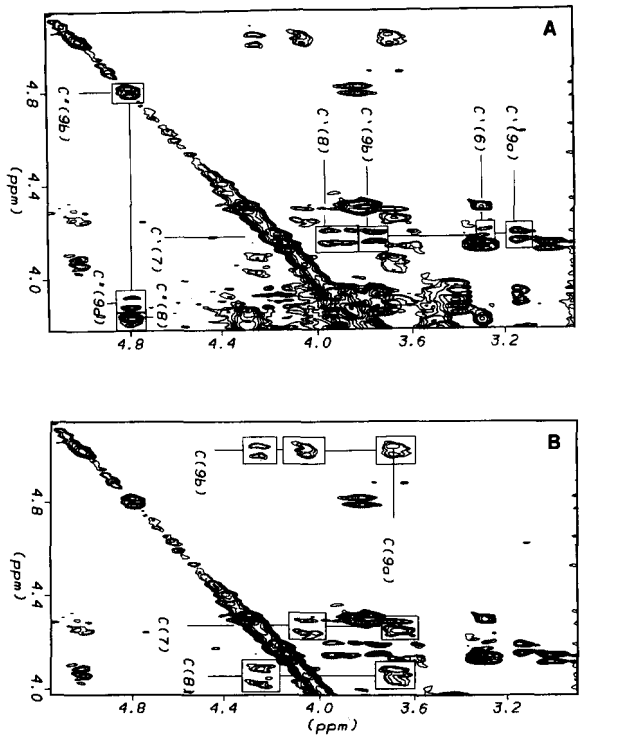


FIG. 9. *A*, plot of the region from the two-dimensional HOHAHA data set containing downfield shifted resonances in monoacetylated $G_{D3}$ species. Resonances corresponding to 7-*O*-Ac $G_{D3}$ are labeled *C'* while resonances corresponding to 9-*O*-Ac $G_{D3}$ are labeled *C''*. *B*, plot of the region from the two-dimensional HOHAHA data set containing downfield shifted resonances in the diacetylated $G_{D3}$ species. Resonances corresponding to downfield shifted protons are labeled *C*.

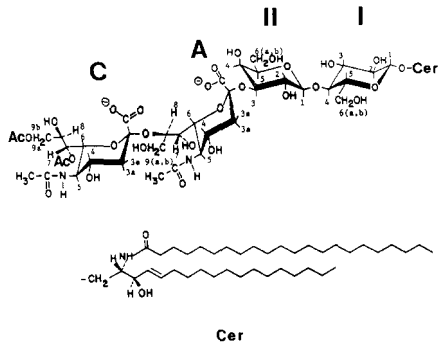


FIG. 10. **The structure of di-*O*-acetylated $G_{D3}$ purified from bovine buttermilk.**

1'-Cer, 7,9-di-*O*-Ac $G_{D3}$). The proposed molecular structure for the di-*O*-acetyl $G_{D3}$ is presented in Fig. 10.

## DISCUSSION

Gangliosides in bovine buttermilk (Table III) are mainly present in the milk fat globule membrane which derives from the apical plasma membrane of the secretory cells in the lactating bovine mammary gland (35, 36). The ganglioside content and composition of the buttermilk have been studied by several groups (21, 33, 37, 38). In the present study, we dialyzed the buttermilk against water. This procedure not only saves organic solvents by removing effectively all small dialyzable substances, which consist of 61% by weight of the buttermilk powder, but also facilitates the recovery and purification of the gangliosides. Our data for ganglioside content and composition are different from that reported by Takamizawa *et al.* (34) who failed to detect any *O*-acetylated ganglioside species. The discrepancy could be due to the relative instability of the *O*-acetylated species which would have been destroyed during the base treatment step used in their ganglioside isolation procedure. Takamizawa *et al.* (34) also reported that about 90% of the gangliosides in bovine buttermilk consisted of hemato-series gangliosides, such as $G_{M3}$, $G_{D3}$, and $G_{T3}$. We found that about 10% of these gangliosides were acetylated on the terminal sialic acid residue. More recently, we reported the presence of *O*-acetyl $G_{D3}$ in bovine buttermilk (21) and suggested that this is a convenient and easily available source for this ganglioside. In the present study, we have extended the previous study and found at least three *O*-acetylated gangliosides in bovine buttermilk.

Since Cheresh *et al.* (14) reported that the monoclonal antibody D1.1 recognized 9-*O*-acetyl $G_{D3}$ on human melanoma cells, which was characterized by alkaline treatment, immunostaining, and periodate oxidation, several groups reported that the alkali-labile ganglioside migrating between $G_{M1}$ and $G_{M2}$ and reacting with mAb D1.1 is 9-*O*-acetyl $G_{D3}$ (15, 21). Constantine-Paton *et al.* (6) found that the antigen recognized by JONES antibody is 9-*O*-acetylated ganglioside $G_{D3}$. In addition to the mAbs D1.1 and JONES, the receptor-destroying enzyme of influenza C virus, sialate *O*-acetylesterase (39), and cancer antennarius lectin (23) can also confirm the presence of 9-*O*-acetylation on the sialic acid residues. More recently, Drazba *et al.* (40) reported that an antigen of the developing chick retina recognized by the monoclonal antibody A2B5 was a 9-*O*-acetylated ganglioside. However, none of them excluded the possibility of two acetyl groups (7 and 9) co-existing in the terminal sialic acid residue of this molecule, nor did they rule out the possibility for the presence of 7-*O*-acetyl $G_{D3}$ which might react with these monoclonal antibodies. In the present study, the purified 7,9-*O*-diacetyl $G_{D3}$ ($G_1$) and a mixture of 7-*O*-acetyl and 9-*O*-acetyl $G_{D3}$ ($G_2$) from bovine buttermilk powder having a similar mobility on the HPTLC, reacted with mAbs D1.1 and JONES. All these species were converted to $G_{D3}$ after mild alkali treatment. However, FAB-mass and NMR spectra suggested that $G_1$ was 7,9-*O*-diacetyl $G_{D3}$ and $G_2$ a mixture of 7-*O*-acetyl and 9-*O*-acetyl $G_{D3}$. It should be pointed out that our resonance assignments are somewhat different from those reported by other workers (16, 22). Many of these previous assignments have relied extensively on well resolved resonances in $^1$H NMR spectra. On the other hand, we have relied extensively on through-bond correlated experiments for our assignment data. Thus, we are not limited to relying solely on the relatively few well resolved resonances in the $^1$H NMR spectra of these *O*-acetylated $G_{D3}$ species. Given the extensive resonance overlap which characterizes the $^1$H NMR spectra of carbohydrates and the additional complication that the $^1$H NMR spectra of *O*-acetylated $G_{D3}$ actually represents a superposition of the $^1$H NMR spectra of the two mono-*O*-acetylated species and the di-*O*-acetylated species, these two-dimensional experiments offer significant advantages in obtaining unambiguous resonance assignments for these compounds.

Although the difference of functional significance between 7-*O*-acetyl $G_{D3}$ in buttermilk and 9-*O*-acetyl $G_{D3}$ in melanoma is not yet understood, it is possible that different positions of *O*-acetylation in ganglioside might play a significant role in cell differentiation and cell growth. Dubois *et al.* (29) reported the presence of alkali-labile ganglioside $G_{T3}$ in 10-day-old chicken embryonic retina and recently confirmed that the ganglioside is 9-*O*-acetylated $G_{T3}$ by using immunostaining with A2B5 and 18B8 monoclonal antibodies. Hirabayashi *et al.* (41) found 9-*O*-acetyl $G_{T3}$ in fetal rat cerebral cortex by using the receptor-destroying enzyme of influenza C virus and

TABLE III

*Gangliosides of bovine buttermilk*

| Ganglioside | Structure | Concentration |
|---|---|---|
| | | *nmol/g dry wt* |
| $G_{M3}$ | NeuAc$\alpha$2→3Gal$\beta$1→4Glc$\beta$1→Cer | 19.98 |
| | Gal$\beta$1→4GlcNAc$\beta$1 | |
| | \| | |
| BG[a] | 6(3)Gal$\beta$1→4Glc$\beta$1→1Cer | 41.40 |
| | \| | |
| | NeuAc$\alpha$2→6Gal$\beta$1→4GlcNAc$\beta$1 | |
| $G_1$ | Ac$_2$-*O*-7,9NeuAc$\alpha$2→8NeuAc$\alpha$2→3Gal$\beta$1→4Glc$\beta$1→1Cer | 0.72 |
| $G_2$ | Ac-*O*-7/9NeuAc$\alpha$2→8NeuAc$\alpha$2→3Gal$\beta$1→4Glc$\beta$1→1Cer | 13.88 |
| $G_{D3}$ | NeuAc$\alpha$2→8NeuAc$\alpha$2→3Gal$\beta$1→4Glc$\beta$1→1Cer | 204.93 |
| $G_3$ | Ac-*O* | 11.87 |
| | \| | |
| | NeuAc$\alpha$2→8NeuAc$\alpha$2→8NeuAc$\alpha$2→3Gal$\beta$1→4Glc$\beta$1→1Cer | |
| $G_{T3}$ | NeuAc$\alpha$2→8NeuAc$\alpha$2→8NeuAc$\alpha$2→3Gal$\beta$1→4Glc$\beta$1→1Cer | 12.73 |

[a] Cited from Ref. 34.

base treatment. The precise chemical structures of these gangliosides, however, have not been carefully examined. In this investigation, we found and characterized the *O*-acetyl $G_{T3}$ in bovine buttermilk by using chemical and immunological methods and provided conclusive evidence that the *O*-acetyl group is present at the terminal sialic acid residue. Because of its reactivity with mAb A2B5, but not with mAb 18B8, it suggests that the position of the acetyl group is most likely to be at the 9- and/or 7-*O*-position on the terminal sialic acid residue. The data also suggest that the free terminal sialic acid residue is important for the interaction of $G_{T3}$ with mAb 18B8. These results are consistent with those reported from chicken embryonic retina (29). Further studies are needed to clarify the position of the *O*-acetyl group in *O*-acetylated $G_{T3}$. Nonetheless, this is also the first report for the presence of *O*-acetyl $G_{T3}$ in bovine buttermilk. Since buttermilk is commercially available, it represents a convenient source for the isolation of this ganglioside.

*O*-Acetylated gangliosides, including 7-*O*-acetyl $G_{D3}$, 9-*O*-acetyl $G_{D3}$, 7,9-di-*O*-acetyl $G_{D3}$, and *O*-acetylated $G_{T3}$ increase the possible number of these molecular species leading to a larger diversity in the pattern of cell surface gangliosides. Although these species have been described originally in developing rat and chicken nervous systems, and human melanomas several years ago, functional studies have been scanty so far. The availability of large quantities of *O*-acetylated gangliosides from bovine buttermilk would facilitate greatly the study of the function of *O*-acetylated gangliosides in cellular differentiation and development.

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