Specific tritium labeling of gangliosides at the 3-position of sphingosines

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Abstract GM1 and GD1a gangliosides, treated with 2,3-dichloro-5,6-dicyano benzoquinone (DDQ) in the presence of Triton X-100 and in a toluene medium were specifically oxidized at the 3-position of sphingosine. The maximum reaction yield (65%) was obtained after 40 hours at 37°C with the following molar ratio of reactants: ganglioside-Triton X-100-DDQ 1:70:125. The formation of the 3-keto derivatives of GM1 and GD1a was demonstrated by: *a*) the appearance of a sharp peak at 1700 cm^{-1} and of a broad band at 1250 cm^{-1} (typical of allylic ketones and of carbonyl groups, respectively) in the infra-red spectrum; *b*) the appearance of an absorption maximum at 230 nm, identical to that featured by 3-keto-cerebrosides, in the ultraviolet spectrum; *c*) the degradation of long chain bases during the process of release from gangliosides and derivatization for analysis by gas–liquid chromatography (expected for long chain bases carrying a keto group in the 3-position); and *d*) the quantitative transformation of 3-keto-GM1 and 3-keto-GD1a to GM1 and GD1a, respectively, upon $NaBH_4$ reduction. Reduction of 3-keto-GM1 and 3-keto-GD1a with [^{3}H]-$NaBH_4$ produced ^{3}H-labeled GM1 and GD1a. [^{3}H]GM1 and [^{3}H]GDla maintained the same carbohydrate and fatty acid composition of the original GM1 and GD1a, and did not contain any saturated long chain bases. Direct proof that the label was at C-3 of long chain bases was given by reoxidation with DDQ, which completely removed the label, and by ozonolysis, after which label was retained on the oligosaccharide-containing fragment. More than 99% of incorporated radioactivity was carried by the long chain bases. The radiochemical purity of labeled gangliosides was greater than 95% and the specific radioactivity was 1.25 and 1.28 Ci/m mol for [^{3}H]GM1 and [^{3}H]GD1a, respectively. —**Ghidoni, R., S. Sonnino, M. Masserini, P. Orlando, and G. Tettamanti.** Specific tritium labeling of gangliosides at the 3-position of sphingosines. *J. Lipid Res.* 1981. **22:** 1286–1295.

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Studies on the physiological role of gangliosides, their interactions with various ligands, and the implications in pathological conditions are in constant progress. Radioactive gangliosides are needed for these studies and the availability of simple procedures for their preparation is of great importance. Four methods have been used: *a*) the biosynthetic method, in which the compound is biosynthesized starting from a radioactive precursor (1, 2); *b*) the reduction method, in which tritium is catalytically added to the double bond of the sphingosine moiety (3, 4); *c*) the galactose oxidase-sodium borohydride reduction method, in which galactose (or N-acetyl-galactosamine) is first oxidized by galactose oxidase, then reduced back with tritiated sodium borohydride (5–8); and *d*) the periodate oxidation-sodium borohydride reduction method, in which sialic acid is specifically oxidized at the level of the side chain, with further reduction with tritiated sodium borohydride (9).

Compounds of relatively low specific radioactivity are obtained by the biosynthetic method; moreover the cost of the procedure is relevant. The reduction method, carried out under conditions of acceptable yield, produces highly radioactive compounds, which, however, due to complete saturation, may display physicochemical properties different from those of the starting material. The galactose oxidase-sodium borohydride reduction method would be advantageous in terms of cost, efficiency, and yield, provided that gangliosides behave as substrates for galactose oxidase. The periodate method, valuable in terms of easy applicability, cannot avoid marked structural modifications of gangliosides.

The purpose of this work was to set up a new procedure for radioactive labeling of ganglioside fulfilling the following requirements: (*a*) wide appli-

Abbreviations: The paper follows the ganglioside nomenclature of Svennerholm (13) and the IUPAC-IUB recommendations (14). GM1, II^{3}αNeuAc-GgOse$_4$-Cer,Galβ1 → 3GalNAcβ1 → 4 (NeuACα2 → 3) Galβ1 → 4Glcβ1 → 1′Cer; GD1a, II^{3}αNeuAc, IV^{3}αNeuAc-GgOse$_4$, NeuAcα2 → 3 Galβ1 → 3GalNAcβ1 → 4 (NeuAcα2 → 3)Galβ1 → 4Glcβ1 → 1′Cer; sphingosines, 2D-amino-octadec-4-en-1,3-D-diol and 2D-amino-eicos-4-en-1,3-D-diol; sphinganines, 2D-amino-octadecan-1,3-D-diol and 2D-amino-eicosan-1,3-D-diol; 3-keto-GM1, oxidized GM1 on the C-3 of sphingosine; 3-keto-GD1a, oxidized GD1a on the C-3 of sphingosine; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; TMS, trimethylsilyl ether; NeuAc, N-acetylneuraminic acid.

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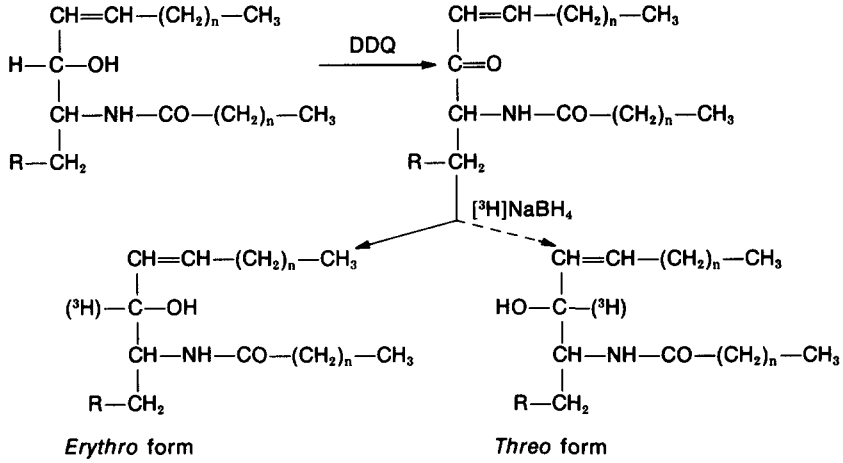

Fig. 1. A general scheme for ganglioside labeling with the DDQ-[^{3}H]$NaBH_4$ method. R, oligosaccharide chain of gangliosides.

cability; (*b*) absence of structural modifications (isotopic); (*c*) high specific radioactivity; and (*d*) good yield.

The approach used (**Fig. 1**) was chemical oxidation of ganglioside at the 3-position of sphingosine with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), a reagent that is specific for allylic hydroxyl groups, followed by reduction with tritiated sodium borohydride. This method was successfully applied to sphingosine, ceramide, and cerebrosides (10–12), but could not be satisfactorily extended to gangliosides (3) because of the formation of side products from sialic acid oxidation. We by-passed this drawback by operating in an organic medium (toluene) containing "inverted mixed micelles" of gangliosides and Triton X-100, a non-ionic surfactant. By the new procedure, gangliosides GM1 and GD1a could be simply and isotopically labeled in the ceramide moiety with high specific activity.

EXPERIMENTAL SECTION

Materials

Commercial chemicals were of analytical grade or of the highest grade available. Common solvents were redistilled before use and water for routine use was freshly redistilled in a glass apparatus. Toluene was dehydrated before use by metallic sodium. Silica gel 100 for column chromatography (0.063–0.2 mm, 70–230 mesh, ASTM), silica gel-precoated thin-layer plates (Kieselgel 60, 250 μm thick, 10 × 20 cm), 2,3-dichloro-5,6-dicyanobenzoquinone, Triton X-100, Triton X-305, Tween 20, and Tween 60 were purchased from Merck GmbH, Darmstadt, West Germany; Triton X-45 and Triton DF-12 were from Rohm and Haas, Philadelphia; Sephadex G-25 fine was from Pharmacia, Uppsala, Sweden; reagents for gas–liquid chromatography were from C.Erba, Milan; glucose, galactose, *N*-acetyl-galactosamine, and m-inositol were from Fluka, Bucks, Switzerland; *N*-acetylneuraminic acid was from Sigma Chem. Co., St. Louis; dialysis tubing was from A. Thomas, Philadelphia; [^{3}H] sodium borohydride (5400 Ci/mol) was from The Radiochemical Centre, Amersham, Great Britain; Dowex 2-X8 (200–400 mesh), prepared in the acetate form according to Svennerholm (15), was from Bio-Rad Laboratories, Richmond, Cal.

GM1 and GD1a gangliosides, extracted from calf brain according to Tettamanti et al. (16), were purified and structurally characterized as described by Ghidoni et al. (17). Their final purity was over 99%.

Methods

Specific oxidation of gangliosides at the C-3 position of long chain bases. The optimal conditions (time and temperature of the reaction; quality and stoichiometry of the reagents) for the specific oxidation of gangliosides at the C-3 position of long chain bases were assessed in preliminary experiments.

The final procedure was as follows. Ten ml of a solution of gangliosides (GM1 or GD1a) (2 mg/ml) in chloroform–methanol 2:1 (by vol) was mixed with 10 ml of Triton X-100 solution (60 mg/ml) in the same solvent. The solvent was evaporated (at 37°C) to dryness under vacuum. The residue was carefully dissolved in 10 ml of DDQ solution (36 mg/ml, in sodium-dehydrated toluene). The mixture was allowed to react at 37°C for 40 hr under continuous stirring in a screw-capped tube; the contents were then evaporated (37°C) to dryness. The dark brown residue was suspended in 10 ml of acetone (in which ganglioside

is not soluble), briefly (15–20 sec) sonicated in an ultrasonic bath, centrifuged at 3000 rpm for 10 min, and the supernatant, containing Triton X-100 and DDQ, was discarded. This treatment was repeated (generally four times) until a clear precipitate was obtained. The oxidized ganglioside, contained in the final precipitate, was further purified by chromatography on a silica gel 100 column (70 × 4 cm), previously equilibrated with chloroform–methanol–water 60:35:7 (by vol), and eluted by the same solvent system. The elution pattern was monitored by TLC as described below. The fractions containing the oxidized ganglioside were collected, evaporated to dryness (37°C), dissolved in few ml of propan-1-ol–water 8:2 (by vol), and stored at 0–4°C. The product was stable under these conditions for at least 6 months.

Characterization of the oxidized gangliosides (3-keto-GM1 and 3-keto-GD1a). The homogeneity of 3-keto-GM1 and 3-keto-GD1a was assessed by TLC as described below; the chemical characterization and purity were established by compositional and spectroscopic analyses (see Analytical procedures). 3-keto-GM1 and 3-keto-GD1a were also reduced back to GM1 and GD1a, respectively, and the resulting compounds were chemically characterized in order to confirm that the only chemical modification that occurred during DDQ treatment was oxidation in the 3-position of long chain bases. For this purpose, 4 mg of oxidized ganglioside dissolved in 15 ml of propanol–water 8:2 (by vol) was treated for 1 hr at room temperature with 10 mg of solid $NaBH_4$ with continuous stirring. No NaOH was added because α,β-unsaturated keto groups are unstable in highly alkaline media.

At the end of the reaction, excess $NaBH_4$ was destroyed and the pH was adjusted to about 6 by adding a few drops of 0.1 N acetic acid. The reaction mixture was then evaporated (37°C) to dryness. The residue was dissolved in 1 ml of water, dialyzed overnight against distilled water, and lyophilized. The residue was chromatographed on TLC plates and chemical analyses were carried out as described for the 3-keto-gangliosides.

Labeling of gangliosides. Solid $[^3H]NaBH_4$ (0.5 mg) was added to 1 mg of oxidized ganglioside dissolved in 3.5 ml of propanol–water 8:2 (by vol). The vial containing the radioactive material was washed with 0.3 ml of the same solution and the wash was added to the mixture.

The mixture was stirred continuously at room temperature for 20 min and then 2 mg of cold $NaBH_4$ was added in order to achieve complete reduction; the mixture was maintained at room temperature for an additional 40 min. At the end of the reaction, 0.1 ml of 0.1 N acetic acid was added, the resulting pH being about 6. The solution was then evaporated (37°C) to dryness, and the residue was dissolved in a few ml of water, and dialyzed overnight at 4°C under a constant flow of distilled water (100 ml/hr).

The labeled ganglioside was purified by chromatography on a silica gel 100 column (70 × 4 cm) previously equilibrated with chloroform–methanol–water 60:35:7.5 (by vol), and eluted with the same solvent. Fractions of 10 ml were automatically collected.

The elution profile was monitored by either TLC or radioscanning. The fractions containing both ganglioside and radioactivity were collected, pooled, and evaporated to dryness. The residue, dissolved in a few ml of propanol–water 8:2 (by vol) was stored at 0–4°C, where it remained fairly stable for at least 6 months.

Ozonolytic oxidation of labeled GM1 and GD1a gangliosides. A sample of each labeled ganglioside (about 2×10^7 dpm) mixed with 5 mg of the same unlabeled ganglioside as carrier, was dissolved in 2 ml of methanol, and submitted to ozonolysis according to Wiegandt and Baschang (18). At the end of the reaction (1 hr), 10 μl of the mixture was chromatographed on a TLC plate (see below) to separate the hydrophilic fragment (containing oligosaccharide) formed by ozonolysis from the hydrophobic fragment (long chain aldehydes, (19)) and the unreacted sphinganine-containing ganglioside-type compounds. After development, the plate was dried, exposed to iodine vapours in order to locate spots, and scanned (see below).

Radiochemical purity and specific radioactivity of labeled gangliosides. The homogeneity of labeled gangliosides was determined by TLC, under the conditions specified below. The radiochemical purity of labeled gangliosides was determined as follows. A sample (ca 10^5 dpm) was mixed with a known amount of carrier ganglioside (as a reference marker). After thin-layer chromatography (see below) the plate was dried, exposed to iodine vapors in order to locate spots, and scanned radiochromatographically using a Packard Radiochromatogram Scanner model 7201.

The specific radioactivity of radiochemically pure gangliosides was determined by assaying radioactivity in a liquid scintillation counter (Packard Tri-Carb 2425) using 5 ml of emulsifier (Instagel, Packard), and by measuring sialic acid by the colorimetric procedure of Warren (20) (see Analytical procedures).

Analytical procedures

Carbohydrate and lipid analysis. Dried samples (0.3 mg) of gangliosides, 3-keto-gangliosides, and reduced

3-keto-gangliosides were methanolyzed in screw-capped tubes with 0.5 ml of 0.5 M anhydrous methanolic HCl at 80°C for 20 hr according to Zanetta, Breckenridge, and Vincendon (21). Fatty acid methyl esters were extracted with 3 × 1.5 ml of n-hexane. Methyl glycosides were analyzed as the corresponding per-O-trifluoroacetyl derivatives (for neutral sugars), or per-N, O-trifluoro-acetyl derivatives (for amino-sugars). These derivatives were prepared by treating dried methyl glycosides in screw-capped tubes with 0.2 ml of a mixture of trifluoroacetic anhydride–dichloromethane 1:1 (by vol) at 150°C for 5 min according to Zanetta et al. (21). The derivatives were analyzed by GLC as described below. By this procedure, the total content of sialic acid was determined as neuraminic acid.

The fatty acid composition of the different products was investigated by GLC analysis of the methyl esters. These were recovered in the n-hexane phase after methanolysis of gangliosides, 3-keto-gangliosides, and reduced 3-keto-gangliosides. After evaporation of the organic phase, the dry residue was dissolved in 20 μl of CS_2 and 2–3 μl was injected directly on the GLC column (see below). Long chain bases were analyzed as the trimethyl-silyl ethers according to the method of Carter and Gaver (22) with some modifications (17). A sample of gangliosides, 3-keto-gangliosides, and reduced 3-keto-gangliosides (1 mg), was hydrolyzed with 0.5 ml of methanol–10 N HCl 9:1 (by vol) for 16 hr at 75°C. After extraction (three times) of the fatty acid methyl esters with n-hexane, the methanolic phase was evaporated under a stream of nitrogen. The dried residue was dissolved in 0.5 ml of 5 N NaOH, and 1.5 ml of water was added after 30 min. The long chain bases fraction was extracted (three times) with 3 ml of diethylether and the organic phase was carefully evaporated in vacuo. The dried residue was submitted to trimethylsilylation (15 min at 60°C) with 10 μl of trimethylsilylimidazole (17). GLC analysis was then performed as described below.

Infrared spectroscopy. Infrared spectroscopy of gangliosides and of their 3-keto derivatives was carried out by a Perkin Elmer infrared spectrophotometer model 621, using the method of solid suspension in KBr.

Ultraviolet spectroscopy. The ultraviolet spectra of 3-keto-GM1 and 3-keto-GD1a were recorded by a Perkin Elmer double beam ultraviolet spectrophotometer, model 550S.

Radio–GLC analysis. A sample of labeled ganglioside (ca 10^5 dpm) was mixed with a known amount (1 mg) of carrier ganglioside and analyzed for individual saccharides, fatty acids, and long chain bases. These determinations were performed by the GLC procedures specified above and peaks corresponding to the various components were radioscanned. A C.Erba chromatograph, model G.V., connected with a Nuclear Chicago flow counter, model 4998, was employed.

Thin-layer chromatography. TLC of gangliosides, 3-keto derivatives, and ozonolyzed products was carried out on silica gel precoated plates under the following conditions: temperature, 18–20°C; solvent system: chloroform–methanol–0.3% aqueous $CaCl_2$ 60:35:8 (by vol); time, 1 hr. Spots were detected by exposure of the plates to iodine vapors at room temperature or by treatment with a p-dimethylaminobenzaldehyde spray reagent followed by heating at 120°C for 10 min (23). The percentage of 3-keto-GM1 and 3-keto-GD1a, formed in the course of oxidation, was determined by densitometric scanning of the TLC plate with a Vitatron densitometer at 650 nm.

Gas–liquid chromatography. GLC of derivatives of individual monosaccharides, long chain bases, and fatty acids was carried out on a C.Erba Fractovap 2400 V instrument (dual-flame ionization detector) equipped with glass columns (200 × 0.3 cm) packed with *a*) 3% SE-30 on Chromosorb W for the analysis of fatty acids and long chain bases, and *b*) 5% OV-210 on Varaport 30 for the analysis of monosaccharides (as trifluoroacetyl (TFA) derivatives). The individual monosaccharides were identified and quantified by using a program at 2°C/min from 120°C to 220°C; the N_2 flow rate was 30 ml/min. The long chain bases were analyzed isothermally at 220°C, at a N_2 flow rate of 50 ml/min, according to Carter and Gaver (22). The fatty acids were identified by using a program at 2°C/min from 170°C to 250°C; the N_2 flow rate was 50 ml/min.

Colorimetric methods. Ganglioside-bound sialic acid was determined by the method of Warren (20) after acid hydrolysis of the sample in 0.05 M H_2SO_4 (1 hr, at 80°C) and purification of liberated sialic acid by ion exchange chromatography on a Dowex 2-X8 (acetate form) column (16). Pure *N*-acetylneuraminic acid was used as the standard.

RESULTS

Optimal conditions for DDQ oxidation of gangliosides

In preliminary experiments, mixtures (at different molar ratios) of GM1 (or GD1a) with each of the tested detergents (Triton X-100, Triton X-45, Triton X-305, Tween 60, Triton DF-12) were dissolved in toluene and allowed to react for different times (up to 60 hr)

TABLE 1. Formation of 3-keto-GM1 by DDQ oxidation: effect of different parameters on the reaction yield

Parameter	Experimental Conditions														
	A				B				C		D				
GM1/Trition X-100 molar ratio	1/10	1/50	1/70	1/90	1/70	1/70	1/70	1/70	1/70	1/70	1/70	1/70	1/70	1/70	1/70
GM1/DDQ molar ratio	1/125	1/125	1/125	1/125	1/15	1/75	1/125	1/150	1/125	1/125	1/125	1/125	1/125	1/125	1/125
Temperature (°C)	37	37	37	37	37	37	37	37	70	70	37	37	37	37	37
Time (hr)	40	40	40	40	40	40	40	40	2	4	10	20	30	40	60
3-keto-GM1	20	40	62	61	35	47	62	57	33	40	38	45	54	62	62
GM1	68	60	38	39	65	53	38	43	59	46	62	55	46	38	38
By-products	12	tr[a]	tr	tr	tr	tr	tr	tr	8	14	tr	tr	tr	tr	tr

3-Keto-GM1, GM1, and by-products were densitometrically assayed as bound NeuAc (p-dimethylaminobenzaldehyde spray reagent) after TLC. Each compound is expressed as percent of total bound NeuAc. In each experimental condition, one parameter was changed; the remainder being kept constant.

[a] tr, Traces.

and different temperatures (from 25 to 70°C) with various amounts of DDQ. Among the detergents used, Triton X-100 provided the most encouraging results. Therefore experiments aimed at establishing optimal conditions for ganglioside oxidation were carried out with this detergent.

As shown in **Table 1,** the amount of 3-keto-GM1, formed from GM1 by DDQ oxidation and determined (as bound NeuAc) by TLC densitometric scanning, was dependent on GM1, Triton X-100, and DDQ concentration, and on temperature and time of reaction. The conditions providing the highest yield of 3-keto-GM1 were the following: ganglioside-Triton X-100-DDQ in a molar ratio 1:70:125 at 37°C for 40 hr. The same conditions also gave the highest yield for GD1a.

The apparent yield of the reaction, as 3-keto derivatives, under optimal conditions, was about 60% (**Fig. 2**) for both GM1 and GD1a, and by-products were present only in trace amounts.

Purification and homogeneity of 3-keto-GM1 and 3-keto-GD1a

As shown in Fig. 2, 3-keto-GM1 and 3-keto-GD1a, purified as described in the experimental section, displayed a single spot on TLC. The degree of their homogeneity can thus be assumed to be greater than 95%.

Characterization of 3-keto-GM1 and 3-keto-GD1a

Compositional analyses of 3-keto-GM1, 3-keto-GD1a, of their corresponding reduced derivatives, and of the starting GM1 and GD1a are reported in **Table 2.** 3-Keto-GM1 and 3-keto-GD1a had the same saccharide and fatty acid composition and the same molar proportions of constituents as the starting GM1 and GD1a gangliosides, respectively. Long chain bases were not detected in 3-keto-GM1 and 3-keto-GD1a; this was likely due to degradation of 3-keto-sphingosine during the derivatization procedure, analogous to what has been shown for 3-keto-ceramides (10). On the other hand, reduced 3-keto-GM1 and 3-keto-GD1a had the same long chain bases composition as the corresponding gangliosides, except that the saturated form (sphinganines) were absent in the reduced keto derivative.

The infra-red spectra of 3-keto-GM1 and 3-keto-GD1a showed a sharp peak at 1700 cm^{-1}, due to the absorption of the allylic ketone, and a broad band at 1250 cm^{-1} typical of carbonyl groups (**Fig. 3**). The infrared spectra of the reduced 3-keto-GM1 and 3-keto-GD1a were exactly the same as those of the corresponding starting gangliosides.

The ultraviolet spectra of 3-keto-gangliosides showed an absorption maximum at 230 nm, which disappeared after reduction. An identical absorption maximum was reported for the 3-keto derivatives of ceramides (10).

Tritiated gangliosides

Both labeled GM1 and GD1a showed a single band by TLC which cochromatographed and had an R_f identical to that of the corresponding original ganglioside. More than 95% of the radioactivity present in the preparation was contained, in both cases, in the corresponding ganglioside, indicating a radiochemical purity better than 95%. The specific radioactivity of the labeled ganglioside was 1.25 Ci/mmol for GM1 and 1.28 Ci/mmol for GD1a.

In order to determine the site of tritiation, labeled

TABLE 2. Saccharide, long chain base, and fatty acid composition of GM1 and GD1a gangliosides, of 3-keto-GM1 and 3-keto-GD1a, and of reduced 3-keto-GM1 and 3-keto-GD1a

	Components													
	Saccharides[a]				Long Chain Bases[b]				Fatty Acids[b]					
Substance	Glc	Gal	GalNAc	NeuAc	C18:0	C18:1	C20:0	C20:1	C16:0	C16:1	C18:0	C18:1	C20:0	C22:0
GM1	1.00	2.11	0.93	0.95	6.3	59.6	2.7	31.4	1.0	0.0	97.0	0.0	2.0	0.0
3-keto-GM1	1.00	2.13	0.91	1.05	0.0	0.0	0.0	0.0	1.1	0.0	96.4	0.1	2.2	0.2
Reduced 3-keto-GM1	1.00	1.98	0.92	1.07	0.0	64.1	0.0	35.9	1.0	0.0	97.1	0.1	1.8	0.0
GD1a	1.00	2.01	0.93	2.04	8.1	53.4	1.0	37.5	2.6	0.8	87.9	1.3	5.2	2.2
3-keto-GD1a	1.00	2.08	0.93	2.11	0.0	0.0	0.0	0.0	2.5	0.7	88.2	1.4	5.1	2.2
Reduced 3-keto-GD1a	1.00	2.08	0.91	2.03	0.0	60.0	0.0	40.0	2.6	0.8	89.0	1.3	4.8	1.5

[a] Relative molar amounts, Glc = 1.00.
[b] As mole %.

GM1 and GD1a were submitted to: *a*) radio GLC analysis of saccharides, fatty acids, and long chain bases; *b*) reoxidation with DDQ; and *c*) ozonolysis, which cleaves the molecules at the double bond (C_4–C_5) in the long chain bases.

When samples of GM1 or GD1a (10^6 dpm) were analyzed by GLC, only peaks of radioactivity that corresponded to TMS derivatives of unsaturated long chain bases (C_{18} and C_{20}) were observed (**Fig. 4**). The ratio between the radioactivity peak areas of the C_{18} and C_{20} sphingosine derivatives corresponded exactly to the ratio of the GLC peak areas of the same compounds. On this basis, it can be concluded that C_{18} and C_{20} compounds had the same specific radioactivity. After injection of 20×10^6 dpm, no peak of radioactivity was observed either in the saccharide or fatty acid derivatives of both gangliosides. Since the minimum radioactivity that could be detected (as a

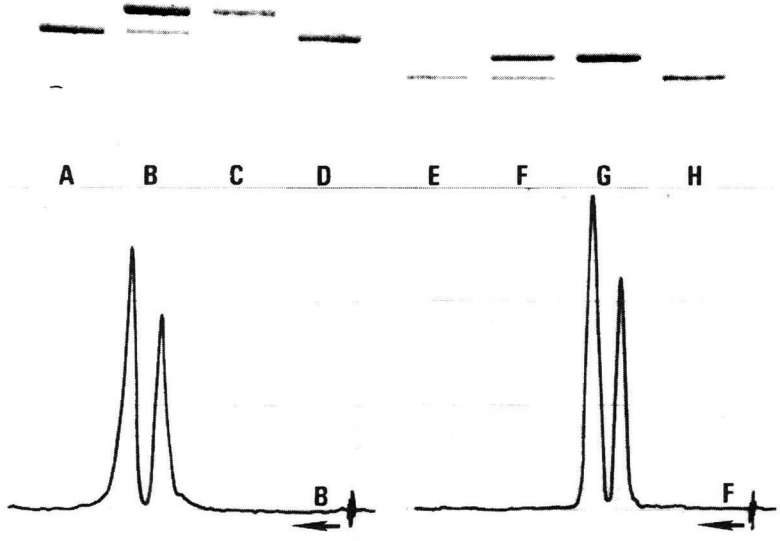

Fig. 2. TLC and densitometric scanning of the products formed by DDQ oxidation of GM1 and GD1a. A, standard GM1 ganglioside; B, the same as A after DDQ oxidation (formation of 3-keto-GM1); C, purified 3-keto-GM1; D, 3-keto-GM1 after $NaBH_4$ reduction; E, standard GD1a ganglioside; F, the same as E after DDQ oxidation (formation of 3-keto-GD1a); G, purified 3-keto-GD1a; H, 3-keto-GD1a after $NaBH_4$ reduction.

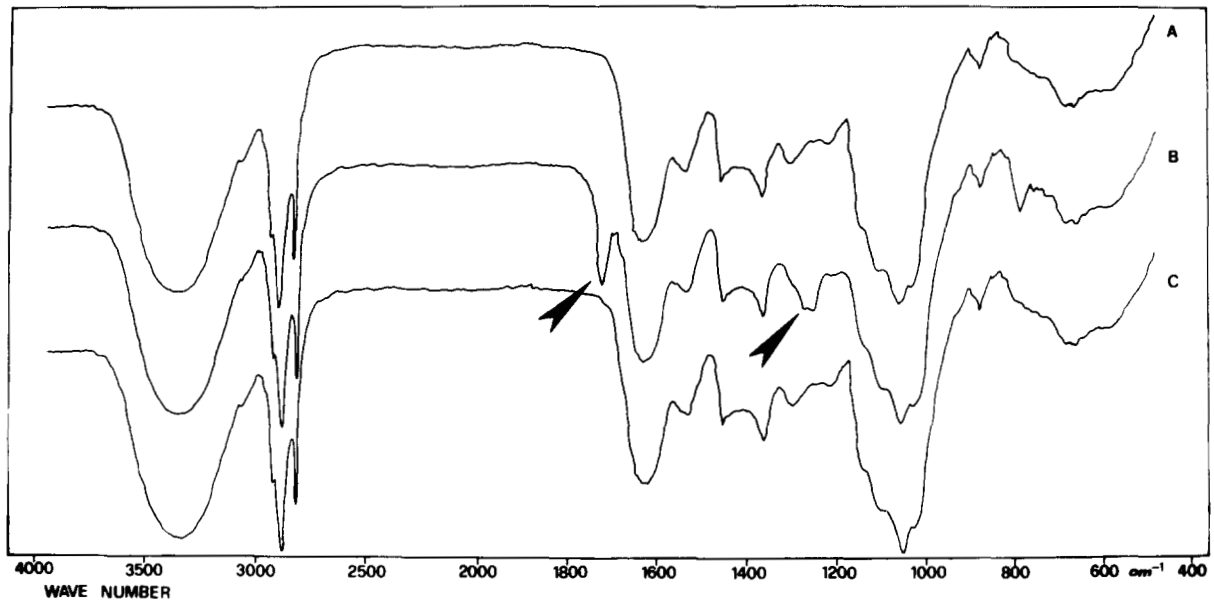

Fig. 3. Infrared spectra of GM1 (A), 3-keto-GM1 (B), and reduced 3-keto-GM1 (C). The spectra corresponding to GD1a (starting compound, 3-keto-GD1a, and reduced 3-keto-GD1a) were quite similar.

peak), under our experimental conditions, was 10^4 dpm, it can be concluded that the radioactivity on saccharides and fatty acids, if present, did not exceed 0.5% of total radioactivity in the labeled gangliosides.

Treatment with DDQ, carried out in order to obtain full re-oxidation of the radioactive compounds, resulted in complete loss of radioactivity. After ozonolysis of the labeled ganglioside, the oligosaccharide-containing fragment, isolated by TLC, retained all the starting radioactivity. Conversely, when the hydrophobic fragments, myristic and palmitic aldehydes, were separated by GLC and radioscanned, no label was detected. This proves that the label was at C-3 of the long chain base.

DISCUSSION

2,3-Dichloro-5,6-dicyanobenzoquinone (DDQ) is a widely employed oxidizing agent (24) which results in selective oxidation of α,β-unsaturated alcohols (25). On this basis Iwamori, Moser, and Kishimoto (11) set up an excellent method for tritiation of sphingosine, ceramide, and cerebroside, consisting of DDQ oxidation at the 3-position of the unsaturated long chain bases, followed by reduction of the obtained 3-keto group with [^{3}H]$NaBH_4$. The extension of this labeling method to more complex glycosphingolipids such as gangliosides has been unsuccessful to date (3).

In the course of pilot experiments, we observed that treatment of gangliosides with DDQ under the same conditions described for cerebrosides (11) produced an abundant series of by-products. The apparent lack of selectivity displayed by DDQ oxidation of gangliosides can be attributed to the presence of sialic acid, which may be an additional site for DDQ action.

It is known that gangliosides, due to their strong amphiphilic properties, undergo micellization in aqueous solution (26–28). Conversely, they are almost completely insoluble in non-polar solvents such as toluene, benzene, hexane, and acetone. Some non-ionic surfactants, such as Triton X-100, which also micellize, form inverted micelles in an organic apolar medium or inverted micellar phases in which the hydrophilic portion of the molecule is included in the aggregate core (29). Since Triton X-100 easily forms mixed micelles with gangliosides (30), we attempted to perform DDQ oxidation in a non-polar system (toluene) containing Triton X-100 and GM1 and GD1a. The results obtained clearly showed that under these conditions a selective oxidation of gangliosides by DDQ could be obtained with no formation of by-products. The success of our approach makes reasonable the hypothesis that the physicochemical state present in our system was an inverted micellar phase of a mixture of ganglioside and Triton X-100. In these aggregates, ganglioside sialic acid must be located in the inner hydrophilic core (inaccessible to the oxidizing agent), while sphingosine C-3 is in the

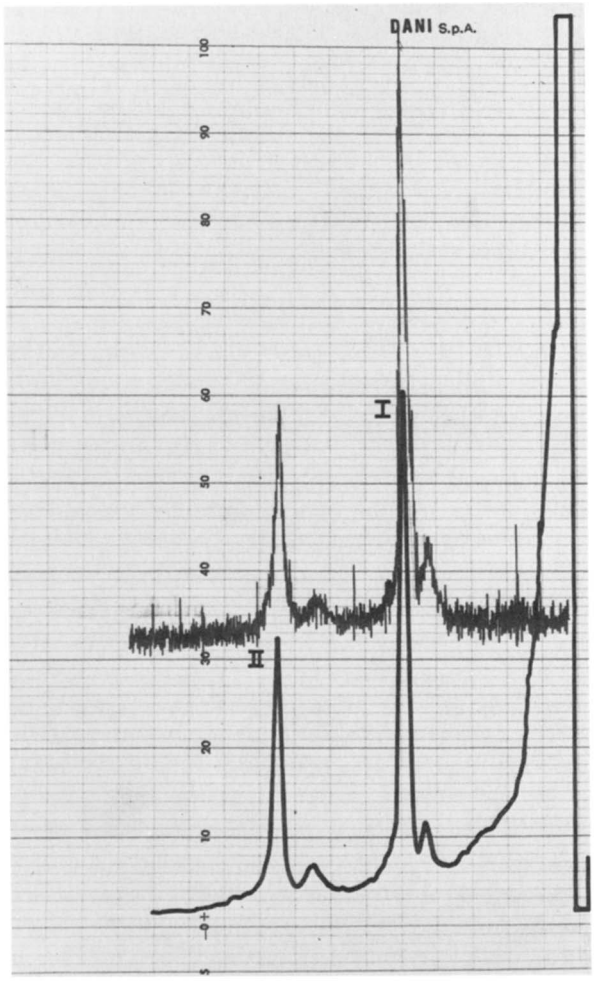

Fig. 4. Radio–GLC analysis of TMS-derivatives of long chain bases, obtained from tritiated GM1. Peaks I and II correspond to C_{18} sphingosine and C_{20} sphingosine, respectively. The corresponding analysis, performed on tritiated GD1a, was similar.

outer region in contact with the organic medium and available to DDQ. The maximum gross yield of ganglioside oxidation was 60%. Considering that about 10% of the starting ganglioside contained saturated long chain bases that cannot undergo oxidation, the net yield of ganglioside oxidation was 65–70%. The incomplete oxidation is probably due to the fact that in toluene the micelles are not free but involved in intermicellar hydrophobic interactions (31) that lead sphingosines to be partially hindered.

Results of compositional and of spectroscopic (infra-red and ultraviolet) analyses performed on the oxidized gangliosides and on the same products after $NaBH_4$ reduction indicated that DDQ selectively oxidized GM1 and GD1a to 3-keto-GM1 and 3-keto-GD1a, respectively. It was observed that long chain bases were not present in oxidized gangliosides, as judged by GLC. Most likely the conditions used for long chain bases release and derivatization (partial acid hydrolysis and extraction with strong alkaline media), when applied to the DDQ-treated gangliosides, led to their complete degradation. This behavior is expected for unsaturated long chain bases with a keto group in the 3-position.

A further finding was that the C_{18} and C_{20} sphinganines present in the starting gangliosides were not present in reduced 3-keto-gangliosides. This is not surprising insomuch as saturated long chain bases do not undergo oxidation with DDQ, and 3-keto-gangliosides with a double bond were separated from non-oxidized species prior to reduction.

Tritiated GM1 and GD1a, obtained by a combination of DDQ oxidation and [^{3}H]$NaBH_4$ reduction, maintained the chemical composition of the corresponding starting gangliosides, with selection of the ganglioside species carrying unsaturated long chain bases (about 90% of the starting gangliosides). All the radioactivity in the labeled gangliosides was at the 3-position of long chain bases. The specific radioactivity of the final compounds equalled that obtained with the best labeling procedures already available (3, 6).

A point to be discussed is the occurrence of structural modifications in gangliosides during the labeling procedure. None were observed, as already mentioned, in saccharides and fatty acids. In addition the long chain bases maintained their double bonds. However reduction of carbonyl groups by $NaBH_4$ is not completely stereospecific and should lead to formation of the *threo*-isomer. Iwamori et al. (11) showed that treatment of cerebrosides with DDQ, followed by $NaBH_4$ reduction, produced a mixture of *erythro* and *threo* diastereoisomers in a molar ratio, 84:16, largely in favor of the *erythro* form. When we tried to study the stereochemistry of sphingosines in natural gangliosides by available methodology (32), we observed that the analytical procedure was able by itself to cause a partial inversion of the *erythro* to the *threo* form. The result was not unexpected since, under the acid conditions used for ganglioside degradation and long chain bases derivatization, the occurrence of an acyl N → O shift (33) and/or the formation of a carbonium ion in 3-position of the long chain bases (34) may actually lead to such a steric inversion. The proportions between the two diastereoisomers changed only slightly when reduced 3-keto-gangliosides were submitted to the same treatment; this may show indirectly that the *threo* form, if any, was present in reduced 3-keto-gangliosides in a small proportion, similar to that reported for cerebrosides (14). Gangliosides are expected to contain the *erythro* form of long

chain bases, and the hydroxyl groups on C-3 are assumed (35) to be involved in hydrogen bond formation with vicinal carbonyl oxygens. It cannot be excluded that in the *threo* configuration the availability of the hydroxyl groups for hydrogen bonding is changed. Thus the precise stereochemistry of long chain bases, and the behavioral differences of the *erythro* and *threo* forms of gangliosides are worthy of being more thoroughly explored. Investigations in this field are already in progress in our laboratory.

In conclusion, the procedure described here, based on DDQ oxidation followed by [^{3}H]$NaBH_4$ reduction, is suitable for isotopical radiolabeling of gangliosides in the 3-position of their unsaturated long chain bases. The final products, of very high specific radioactivity, are easily obtained in good yield. Since the specificity of the oxidation is dependent upon formation of inverted micellar phases, the procedure should be suitable for radiolabeling all naturally occurring gangliosides.

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