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OXIDATIVE DEGRADATION OF GLYCOSPHINGOLIPIDS REVISITED: A SIMPLE PREPARATION OF OLIGOSACCHARIDES FROM GLYCOSPHINGOLIPIDS*

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ABSTRACT

In order to cleave gangliosides and isolate the oligosaccharide portion, the allylic nature of OH-3 of the sphingenine base was utilized in its selective oxidation to a ketone group by 2,3-dichloro-5,6-dicyanobenzoquinone. Triethylamine treatment of the oxidation products resulted in the β-elimination of the intact oligosaccharide. The isolation of the pure oligosaccharide from the modified ceramide residue and unreacted ganglioside was obtained by liquid chromatography. Preliminary investigations suggest that the same reaction conditions can be used for an analogous elimination of oligosaccharides linked to the serine or threonine residues of glycoproteins.

INTRODUCTION

The oligosaccharide component of glycolipids and glycoproteins have been implicated as receptors for lectins[1], bacterial toxins[2], and viruses[3]. They have also been reported to function in cell–cell adhesion[4], in the determination of the half-life of circulating sialoglycoconjugates[5], and as regulators of cell growth[6]. To determine the role(s) of the oligosaccharide component in these biological reactions, one has to be able to separate it from the lipid or protein component, in unchanged, pure form, and in good yield.

During studies of various ganglioside structures, several procedures were developed for the cleavage and purification of the oligosaccharide portion of glycosphingolipids[7–9]. In each method, the initial reaction was the oxidative cleavage of the C-4–C-5 double bond of the sphingosine base[7–9]. The free, intact oligosaccharide was then released by treatment with alkali.

We have developed a simpler and more straightforward procedure for the cleavage and isolation of the oligosaccharide portion of glycosphingolipids. This

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consists of the selective oxidation of the allylic OH-3 of the sphingenine base. Then, the oligosaccharide can be cleaved from the 3-ketosphingolipid intermediate by a base-catalyzed β-elimination.

EXPERIMENTAL

Materials. — Gangliosides were isolated from the gross gray matter of bovine brains by the procedure described by Folch-Pi *et al.*[10]. Individual ganglioside components were isolated by chromatography on silica gel in a chloroform–methanol–water gradient of increasing polarity[11]. The isolated gangliosides were identified by t.l.c. (under pressure) on plates obtained from Merck (Darmstadt, West Germany) by use of the procedure of Ghidoni *et al.*[12], and were detected with the resorcinol spray[13], and quantitatively determined with a Schoeffel SD 3000 scanning spectrodensitometer. 2,3-Dichloro-5,6-dicyanobenzoquinone (DDQ) was recrystallized from benzene prior to use. Triethylamine was freshly distilled and anhydrous toluene was obtained by distillation and drying over Na.

Oxidation of gangliosides. — A 2:3 molar mixture of G_{M1} (ref. 14) and G_{D1a} (40 mg) was oxidized with DDQ by the procedure described by Ghidoni *et al.*[12]. The reaction was monitored by l.c., and terminated after 22 h.

Base-catalyzed elimination of oligosaccharides from oxidized gangliosides. — The mixture of oxidized G_{M1} and G_{D1a} was dissolved in 1:1 methanol–water (6 mL), triethylamine (0.6 mL) was added, and the mixture was kept at 50° for 90 min. At the end of this time, it was dried at room temperature under a stream of N_2, and the residue was dissolved in methanol (4 mL) and examined by t.l.c.

Isolation of pure oligosaccharides by l.c. — Oligosaccharides were separated from gangliosides containing unreactive sphinganine base and the modified ceramide residues formed during the elimination reaction by preparative l.c. The mixture obtained after base treatment (0.5-mL aliquots) was injected onto a Zorbax C8 preparative column (21.2 mm × 25 cm) (DuPont Co., Wilmington, DE) and eluted with a linear gradient (30 min) of 1:1 methanol–water as the initial solvent and pure methanol as the final solvent. The flow-rate was 4.5 mL/min. The effluent was monitored with a Schoeffel Spectroflow Monitor SF 770 at a wavelength of 248 nm, and with a Waters Differential Refractometer R 401. Individual oligosaccharides were obtained by l.c. of the isolated oligosaccharide mixture on a PSM 60-S column (6.2 mm × 25 cm; DuPont Co., Wilmington, DE) and elution with pure methanol at a flow rate of 0.225 mL/min; the effluent was monitored as just described, except at a wavelength of 190 nm.

Characterization of oligosaccharides. — The presence of a reducing sugar in the isolated oligosaccharides was determined by the procedure of Meyer and Larner[15]. Bound sialic acid was assayed according to the method described by Warren[16]. Appropriate control experiments were used to monitor the presence of free sialic acid. D-Glucose concentration was determined by the D-glucose oxidase procedure for free D-glucose on samples that had been hydrolyzed with M HCl at

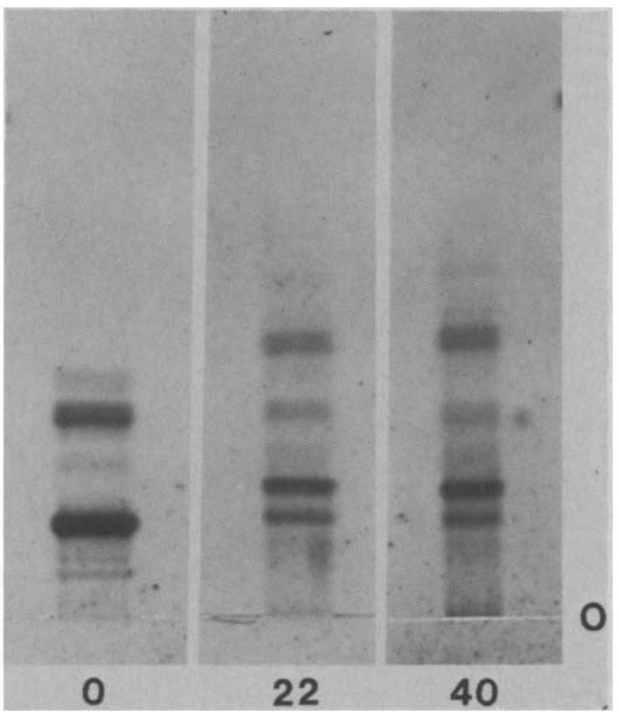


Fig. 1. T.l.c. of a mixture of G_{M1} and G_{D1a} prior to exposure to DDQ (0), and after exposure to DDQ for 22 h (22) and for 40 h (40). The O to the right of the figure indicates the origin. The plate was developed[12] in 60:35:8 chloroform–methanol–0.3% $CaCl_2$ and the spots were detected[13] with resorcinol.

100° for 16 h. The presence of at least two sialosyloligosaccharides was determined by t.l.c. using 60:30:1 methanol–acetic acid–water as the solvent (empirically determined to be effective) and resorcinol to detect the separated components.

RESULTS AND DISCUSSION

Reaction of the gangliosides with DDQ for either 22 or 40 h resulted in the same degree of oxidation of G_{M1} and G_{D1a}. This was determined by comparison of the amount of oxidized and unoxidized compounds (determined by spectrodensitometric-scanning of resorcinol-positive bands) after separation by t.l.c. (Fig. 1). Sixty-six percent of the G_{D1a} was oxidized after either 22 or 40 h. At the end of 22 h, 55% of the G_{M1} had been oxidized, a value that was essentially unchanged

TABLE I

RELATIVE AMOUNTS OF SIALIC ACID-CONTAINING COMPONENTS ASSOCIATED WITH FREE OLIGOSACCHARIDES AND WITH LIPID COMPONENTS

Time (h)	*Sialic acid[a] (%) associated with*		
	Oligosaccharide	G_{D1a}	G_{M1}
1.5	68	23	8
2.5	68	23	9
3.5	66	24	11
6.0	71	19	10

[a]Sialic acid concentration was determined by spectrodensitometric scanning of the resorcinol-positive bands at 578 nm.

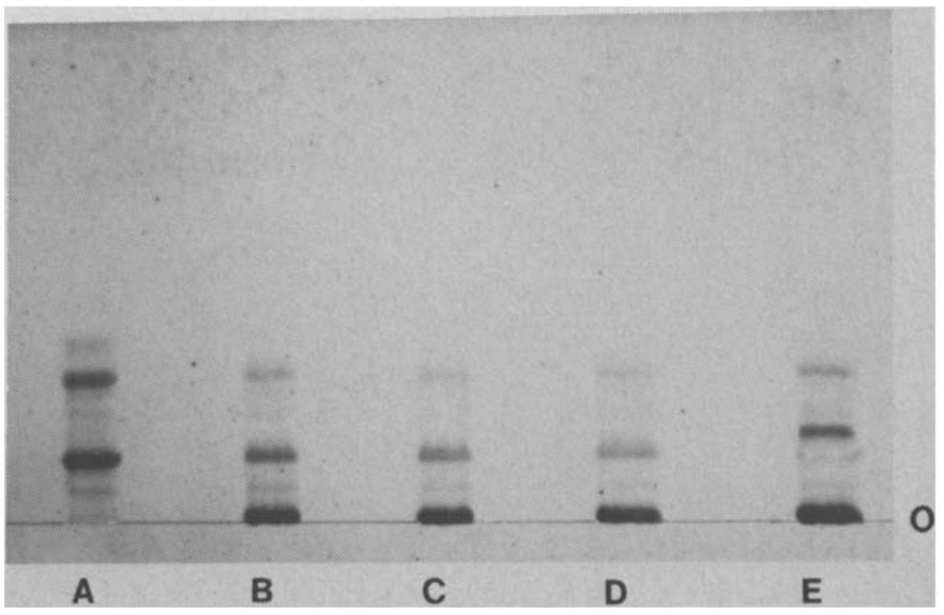


Fig. 2. T.l.c. of G_{M1} and G_{D1a} after oxidation by DDQ (A) and reaction with triethylamine at 50° for: 1.5 (B), 2.5 (C), 3.5 (D), and 6 (E) h. The O to right of the figure indicates the origin. The plate was developed[12] in 60:35:8 chloroform–methanol–0.3% $CaCl_2$ and the spots were detected[13] with resorcinol.

(50%) at the end of 40 h. Hence, the ganglioside samples were oxidized for 22 h.

Triethylamine-catalyzed elimination of the oligosaccharide residues from the oxidized gangliosides was essentially complete after 1.5 h (see Table I), as monitored by the apperance of resorcinol-positive material (free sialosyloligosaccharide) at the origin after developing the t.l.c. plate; the remaining ganglioside components had much higher R_F values (Fig. 2). No further change in pattern was observed for samples exposed to triethylamine for up to 6 h. The analysis of oligo-

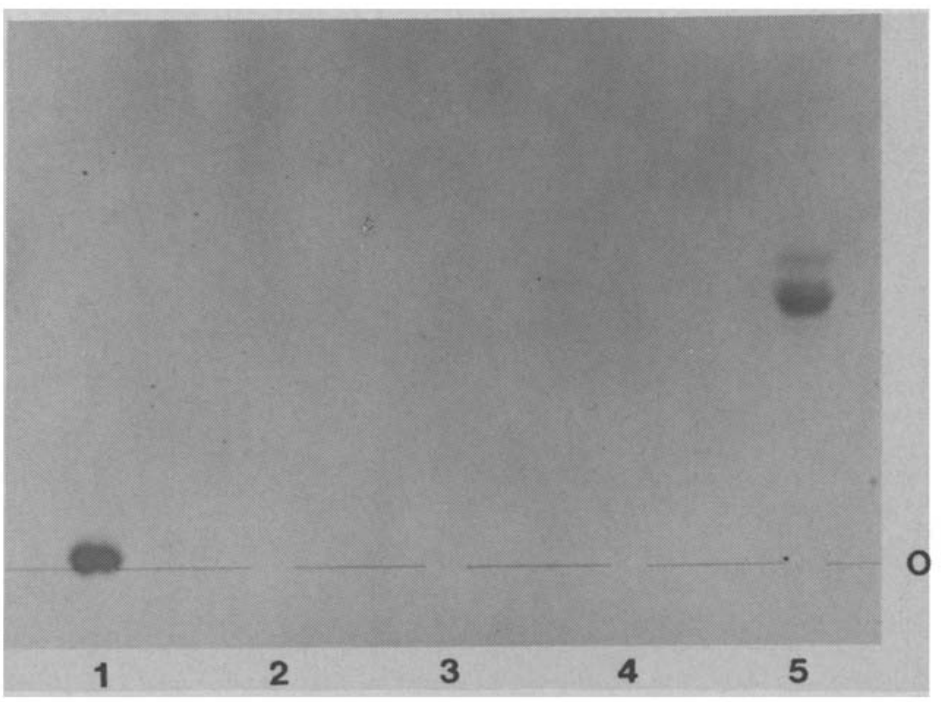


Fig. 3. T.l.c. of the fractions recovered upon l.c. of the DDQ- and triethylamine-treated G_{D1a} on a Zorbax C8 column. Fraction 1 was eluted between 7 and 13 min, and fraction 5 after 29 min. The O to right of the figure indicates the origin. The plate was developed[12] in 60:35:8 chloroform–methanol–0.3% $CaCl_2$ and the spots were detected[13] with resorcinol.

saccharide release at shorter time-intervals indicated that little change occurred after 30 min. However, the 1.5-h time interval was routinely used.

T.l.c. indicated that the oligosaccharides were eluted from the Zorbax C8 column between 7 and 9 min (Fig. 3). None of the lipid components were eluted prior to 19 min, therefore an apparently pure preparation of lipid-free oligosaccharide was recovered. Identification of the number of oligosaccharide components present was made by t.l.c. using 60:30:1 methanol–acetic acid–water as the developing solvent (Fig. 4). Only bound sialic acid was found in association with the recovered oligosaccharides (thiobarbituric acid assay[6]), the presence of a reducing sugar was indicated by the positive silver nitrate reaction[15], and the presence of bound glucose was ascertained by the glucose oxidase assay (no reaction was obtained for unhydrolyzed samples).

The procedure described herein took advantage of the heightened susceptibility of the allylic OH-3 of the sphingenine base towards oxidation. This permitted the selective oxidation of OH-3 to the corresponding keto group without affecting the rest of the molecule. The presence of the carbonyl group at C-2 resulted in the activation of H-2, thereby making it susceptible to base which resulted in the elimination of the intact oligosaccharide portion of the molecule (see Scheme 1).

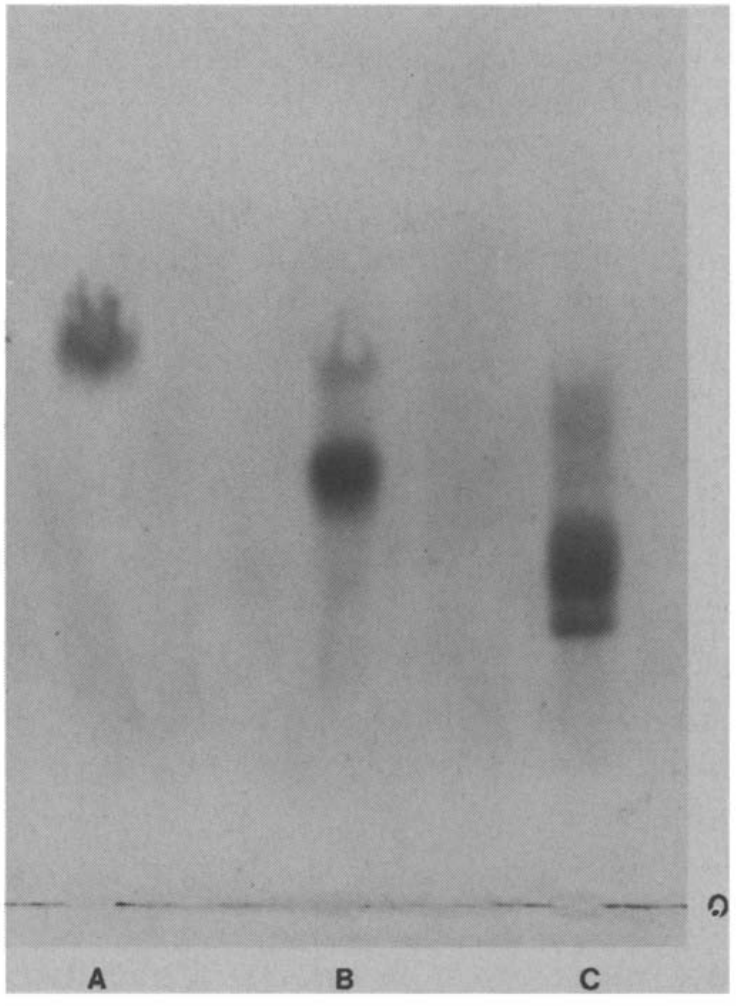
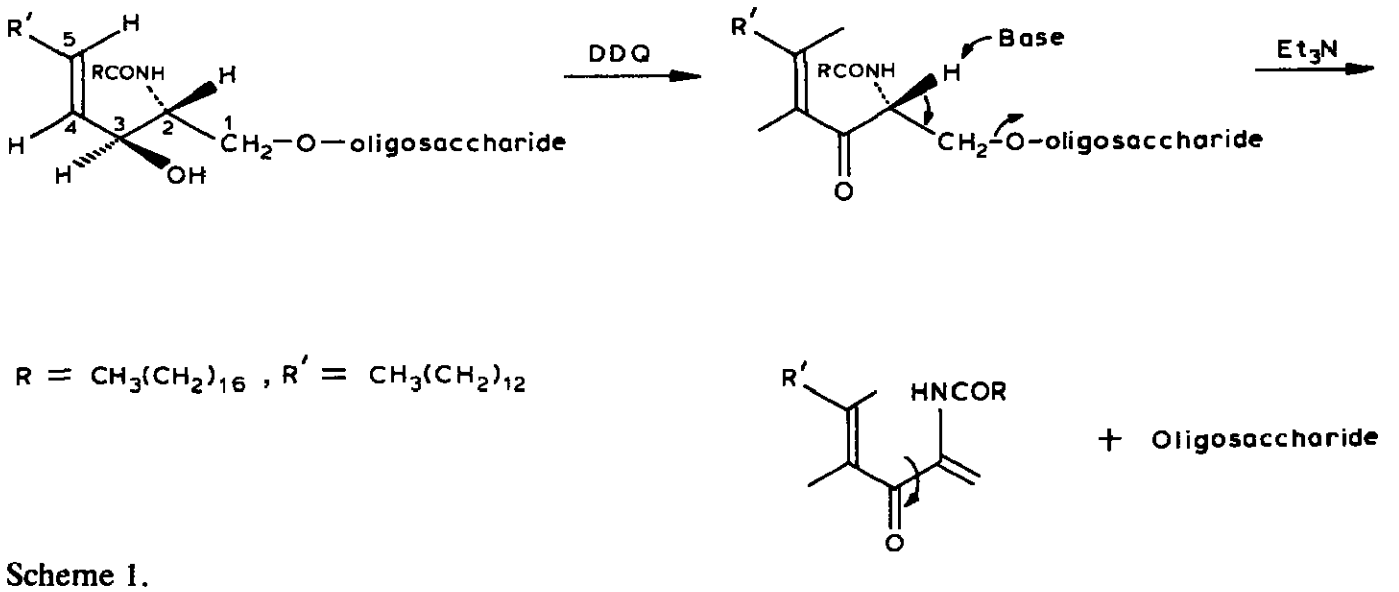


Fig. 4. T.l.c. of sialyllactose (A), the oligosaccharide isolated from G_{M1} (B), and that isolated from G_{D1a} (C). The O to the right of the figure indicates the origin. The plate was developed in 60:30:1 methanol–acetic acid–water and the spots were detected[13] with resorcinol.



Scheme 1.

Characterization of the recovered products suggests that intact oligosaccharides were recovered. All of the sialosyl residues were bound (unhydrolyzed material gave no color in the Warren reaction[16]), a reducing group was present (presumably due to the unchanged D-glucose residue), and the difference in mobility upon t.l.c. indicated that two different oligosaccharides were recovered when a mixture of G_{M1} and G_{D1a} was used.

Previous procedures[7–9] have utilized the oxidative cleavage of the C-4–C-5 double bond of the sphingosine base as the first step. The sphingenine fragment containing the oligosaccharide and fatty acid residues, obtained after cleavage of the double bond, consisted of either a tetrose (formed from the C-1–C-4 portion of sphingosine) or a triose (formed from the C-1–C-3 portion of sphingosine). Treatment with alkali (such as sodium hydroxide or sodium carbonate) then liberated the intact oligosaccharide, residue from these intermediates. In the oligosaccharide-triose intermediate the H-2 is in the α-position to the aldehyde group and the subsequent oligosaccharide elimination occurs as described above. In the oligosaccharide-tetrose intermediate, H-2 is in the β-position with regard to the aldehyde group and, thus, not susceptible to attack by a base. However, the base-catalyzed isomerization of the tetrose into the tetrulose (Lobry de Bruyn–Alberda van Ekenstein transformation), which presumably takes place, permits the eventual elimination of the oligosaccharide in this case as well.

In addition to being less direct, the previous procedures employed for the oxidation of the sphingosine double bond present practical problems. Ozonation requires an ozonizer; osmium tetraoxide is very toxic; and 3-chloroperoxybenzoic acid degradation proceeds *via* a more complex reaction sequence. Oxidation with either osmium tetraoxide–periodate or with 3-chloroperoxybenzoic acid also requires the peracetylation of the oligosaccharide residue prior to oxidation. The use of DDQ to oxidize the sphingosine OH-3 has no effect on the rest of the glycosphingolipid molecule, hence no derivatization is needed. Finally, the use of triethylamine as the base eliminates the necessity of removing sodium ions.

The oxidation of the gangliosides by DDQ, followed by elimination with tri-

ethylamine, produced a u.v. absorbent product which was monitored at a wavelength of 248 nm. The oligosaccharides did not absorb light at this wavelength but had a positive refractive index. The u.v.-absorbing product could be almost completely separated from the oligosaccharide on the preparative Zorbax C8 column, the oligosaccharide being eluted just prior to the u.v.-absorbing material. Some overlap occurred under the conditions used, and the oligosaccharide eluted at that point was not analyzed. The Zorbax PSM 60-S column provided an excellent separation of the u.v.-absorbing material from the oligosaccharide. The u.v.-absorbing material was eluted first from the column, behaving as a molecule of greater molecular-weight than the oligosaccharide.

We believe that the method described herein provides a simple and direct procedure for the isolation of the oligosaccharide component of glycosphingolipids. Preliminary results indicate that the same reaction conditions can also induce an analogous elimination reaction of the oligosaccharide chain linked to a serine or threonine residue of glycoproteins. Triethylamine treatment of bovine submaxillary mucin resulted in the release of an oligosaccharide which migrated just above sialyl-lactose upon t.l.c. (as might be expected for a Sia→GalNAc disaccharide) and contained a reducing sugar. The technique described provides a method for obtaining specific oligosaccharides that can be used in studies designed to elucidate their role(s) in reactions in which the glycosphingolipid (and possibly glycoprotein) from which they are derived is implicated.

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