ASBMB JOURNAL OF LIPID RESEARCH

notes on methodology

An improved method for the covalent attachment of glycolipids to solid supports and macromolecules

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Summary A simplified method is presented for the oxidation of the olefinic bond of the sphingosine moiety of glycosphingolipids to a carboxyl group. Coupling of such "glycolipid acids" to glass beads, agarose gels, proteins, and polyacrylic hydrazide polymers is described. Solid supports and macromolecules that have been derivatized in this fashion are useful reagents for a variety of studies in cell biology and immunology.—**Young, W. W., Jr., R. A. Laine, and S. Hakomori.** An improved method for the covalent attachment of glycolipids to solid supports and macromolecules. *J. Lipid Res.* 1979. **20:** 275–278.

Supplementary key words crown ether · poly-glycolipid · glycolipid acid · Forssman glycolipid

The covalent attachment of glycosphingolipids to solid supports and macromolecules provides reagents that are useful for a variety of investigations. These include: *1*) interaction of glycolipid carbohydrate determinants with cell surfaces; *2*) affinity purification of glycosylhydrolases or transferases that are specific for glycolipids; *3*) purification of anti-glycolipid antibodies; *4*) production of immune responses against antigens containing multivalent glycolipid determinants; and *5*) radioimmunoassay of both glycolipids and antiglycolipid antibodies.

A procedure was described previously for the coupling of glycolipids to solid supports (1). That method utilized oxidative ozonolysis of the olefinic bond of the sphingosine moiety of the glycolipid to yield carboxyl-bearing products which were then coupled to the amino groups of the solid supports. In the present simplified method, oxidation of the olefinic bond to the carboxyl function is accomplished with potassium permanganate which has been solubilized in benzene by the crown ether dicyclohexyl-18-crown-6 (2, 3). This note also describes the synthesis of "poly-glycolipids" by the covalent attachment of glycolipid acids to polyacrylic hydrazide.

MATERIALS AND METHODS

Purified glycolipids were prepared from the following sources according to established procedures (4): ceramide dihexoside and globoside from human erythrocytes, N-acetylhematoside from dog erythrocytes, gangliotriosyl ceramide (asialo GM_2) from guinea pig erythrocytes, and Forssman glycolipid from goat erythrocytes.

Preparation of glycolipid acids

The carboxyl group of N-acetylhematoside was first protected by forming the methyl ester (5). Purified hematoside, 20 mg, was dissolved in 20 ml of methanol followed by the addition of 200 mg of Dowex 50-H^+ form. The reaction mixture was stirred at room temperature for 1–3 days until methyl ester formation was complete as judged by thin-layer chromatography.

The following procedure applies both to neutral glycolipids and to ganglioside methyl esters; hematoside is shown as an example in **Fig. 1.** Twenty mg of hematoside methyl ester was acetylated overnight at room temperature with 0.8 ml of pyridine and 0.6 ml of acetic anhydride (6). Reagents were removed by rotary evaporation using an excess of toluene until the odor of acetic anhydride was absent. Acetylated glycolipid was dissolved in 1 ml of benzene. $KMnO_4$, 20 mg, was added, followed by the addition of 90 mg (6 drops) of the crown ether, dicyclohexyl-18-crown-6 (Aldrich Chemical Co., Inc., Milwaukee, WI). The reaction mixture was stirred vigorously at room temperature for about 90–120 min, until all traces of purple color were absent and had been replaced by the brown MnO_2 residue. After adding 1 ml of acetone, the mixture was applied directly to a Sephadex LH20 column (Pharmacia; 1.4 by 40 cm prepared in benzene–acetone 1:1). The column was eluted with benzene–

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Abbreviations: glycolipid acid, 2-hydroxy-3-(N-fatty acylamido)-4-glycosyl-butanoic acid; ceramide dihexoside, Gal(β1-4)GlcCer; N-acetylhematoside, NeuNAc(α2-3)Gal(β1-4)GlcCer; gangliotriosyl ceramide (asialo GM_2), GalNAc(β1-4)Gal(β-1-4)GlcCer; globoside, GalNAc(β1-3)Gal(α1-4)Gal(β1-4)GlcCer; Forssman glycolipid, GalNAc(α1 - 3)GalNAc(β1 - 3)Gal(α1 - 4)Gal(β1 - 4)GlcCer; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

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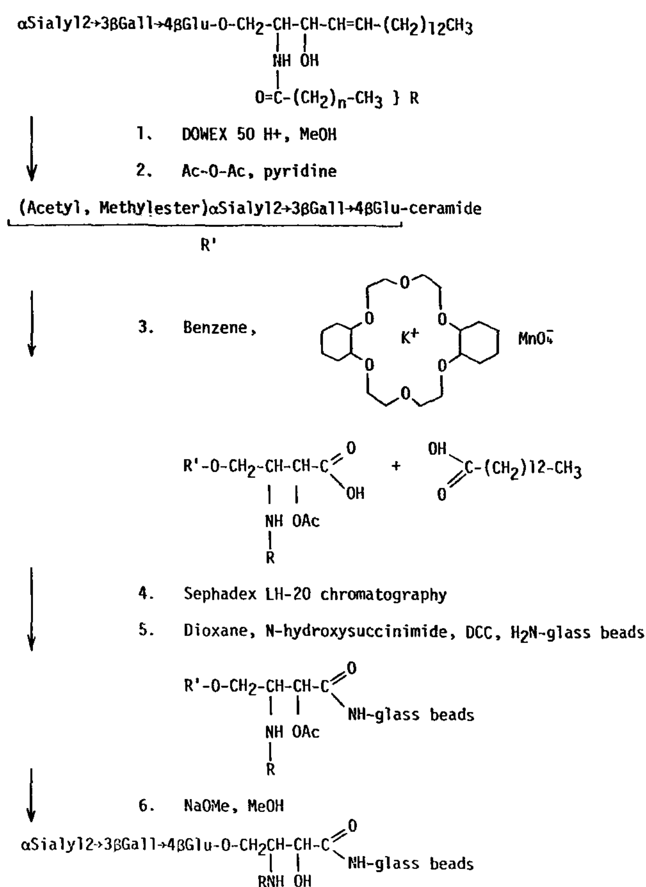

Fig. 1. Synthesis of hematoside–glass beads complex.

acetone 1:1, and the orcinol-positive fractions were pooled. These fractions which contained the acetylated glycolipid acid appeared just ahead of the strongly iodine-positive, orcinol-negative fractions which contained the crown ether and free fatty acids.

In some cases the acetylated glycolipid may not be quantitatively oxidized, due to coating of unreacted potassium permanganate with manganese dioxide. In this event the reaction mixture can be filtered with Celite filter aid (Johns Manville, Lompoc, CA) to remove MnO_2 and then, after the addition of 10 mg of $KMnO_4$, the reaction can be continued. Any unreacted acetylated glycolipid that remains after this second incubation probably does not contain a susceptible olefinic bond in its ceramide moiety.

An alternative procedure for the separation of acetylated glycolipid acid and crown ether has also been devised which avoids the use of the rather expensive Sephadex LH20. Acetylated globoside was oxidized as described above, and then the benzene was removed under a N_2 stream. The residue was dissolved in diethyl ether–ethanol 95:5 and applied to a Biosil A column (Biorad Laboratories, Richmond, CA; 2.5 by 5 cm) prepared in the same solvent. The crown ether material eluted with diethyl ether–ethanol 9:1 while the acetylated oxidized globoside eluted with 100 ml of diethyl ether–ethanol 1:1.

The yield of glycolipid acid from this procedure was as follows: ceramide dihexoside 59%, asialo GM_2 47%, *N*-acetylhematoside 57%, and globoside 56%. The glycolipid acid structure was examined by combined gas–liquid chromatography and mass spectrometry after methanolysis and *N*-acetylation as previously described (1). A characteristic peak representing the degradation product of the sphingosine moiety was identified as 2,4-dihydroxy-3-aminobutyric acid.

Coupling glycolipid acid to glass beads

Ten mg of acetylated glycolipid acid was dissolved in 1 ml of dioxane in a small, screw-capped tube. The activated glycolipid ester was formed by adding 5 mg of *N*-hydroxy succinimide dissolved in 0.5 ml of dioxane plus 5 mg of dicyclohexyl carbodiimide (Sigma Chemical Co., St. Louis, MO) in 1 ml of dioxane and incubating at 15°C for 30 min. Then 1 g of alkylamine glass beads (CPG-1350 Corning; Pierce Chemical Co., Rockford, IL; 60 μeq NH_2 groups per gram) and 5 ml of dioxane were added, followed by mixing on a tilting table at room temperature for 72 hr. One drop of glacial acetic acid was added to derivatize the remaining amino groups by continued incubation for 24 hr.

The product was washed with dioxane and then chloroform–methanol 2:1. To deacetylate the attached glycolipid (and also to remove the methyl ester of ganglioside sialic acid), the glass beads were suspended in 10 ml of chloroform–methanol 2:1 plus 2 ml of 0.5% sodium methoxide in methanol (5). After 30 min at room temperature, the beads were filtered and washed with 50 ml of chloroform–methanol 2:1, followed by washing with 100 ml of water, and 50 ml of phosphate buffer, pH 7.0. The beads were stored in this buffer after addition of sodium azide to 0.02%. Evidence of coupling was obtained by methanolysis of the product as previously described (1).

Coupling glycolipid acid to agarose gel

Neutral glycolipid acid was first deacetylated with sodium methoxide as described above. Then the glycolipid acid was dissolved in 4.0 ml of dimethylformamide and mixed with 4.0 ml of packed aminoethyl-Sepharose (7). The pH was adjusted to 4.7 with 1 N HCl. Fifty mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was dissolved in 0.5 ml of water and added with mixing over 5 min. After 20 hr at room temperature, the Sepharose gel was washed

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with 200 ml of 50% dimethylformamide in water in a filter funnel, and then transferred to a column for further washing with distilled water and finally with phosphate buffer pH 7.0 until no more products were eluted. The column was stored in the presence of 0.02% sodium azide. Using gangliotriosyl ceramide labeled with tritium (8), 38% of the glycolipid acid remained bound to the Sepharose gel. This represented 130 nmol glycolipid bound per ml of Sepharose gel.

Using this procedure glycolipid acids can be coupled readily to agarose gels that bear free amino groups. However, the glycolipid acid must be deacetylated before coupling because the agarose gel cannot withstand the basic conditions required for deacetylation. Therefore, coupling to agarose is not possible for ganglioside acids, which must be coupled as acetylated, methyl ester derivatives to avoid coupling via the sialic acid carboxyl group. However, it may be possible to overcome this limitation by using recently introduced agarose derivatives such as Sepharose CL (Pharmacia) which are stable to a wide pH range.

Coupling glycolipid acid to protein

Neutral glycolipid acid was first deacetylated as described above. One mg of glycolipid acid was dissolved in 0.5 ml of dioxane–water 1:1, and the activated glycolipid ester was formed by adding 1 mg of *N*-hydroxysuccinimide and 20 mg of EDC, both dissolved in dioxane–water 1:1. After incubation at 15°C for 30 min, 1–5 mg of the protein was added in the same solvent. Incubation was continued at 4°C for 20 hr with gentle mixing, and then the product was dialyzed extensively against distilled water. The extent of coupling can be determined by assaying the product for protein using both the assay of Lowry et al. (9) and the fluorescamine assay (10). The former assay is independent of the number of free amino groups while the latter directly measures free primary amines; hence, the difference in values indicates the extent of coupling of protein amino groups.

Synthesis of poly-glycolipids

Polyacrylic hydrazide was prepared according to the procedure of Wilchek and Miron (11). Lyophilized polyacrylic hydrazide was recrystallized from methanol–glacial acetic acid 500:1 (v/v), redissolved in water, and the small amount of insoluble material was removed by filtration. The filtrate, after lyophilization and resuspension in water, contained 6 mmol of hydrazide groups per gram as determined by the 2,4,6-trinitrobenzene sulfonate assay (12).

Forssman glycolipid acid was prepared as described above. By thin-layer chromatography in the solvent dichloroethane–methanol 8:2 (v/v), the acetylated Forssman acid had an R_f value of 0.39 while acetylated Forssman gave 0.78. After deacetylation as described above, Forssman acid had an R_f of 0.14 in the solvent chloroform–methanol–water 60:35:8 whereas Forssman gave 0.34. Forssman acid, 1.5 mg, was dissolved in water and mixed with 1 mg of *N*-hydroxysuccinimide and 10 mg of EDC at 15°C for 30 min. The resulting activated ester was coupled to 5 mg of polyacrylic hydrazide by overnight incubation at 4°C. After extensive dialysis against distilled water, unreacted hydrazide groups were derivatized by the addition of 0.1 ml of formaldehyde. After 15 min at 4°C, 40 mg of $NaBH_4$ was added for Schiff base reduction. The product was extensively dialyzed.

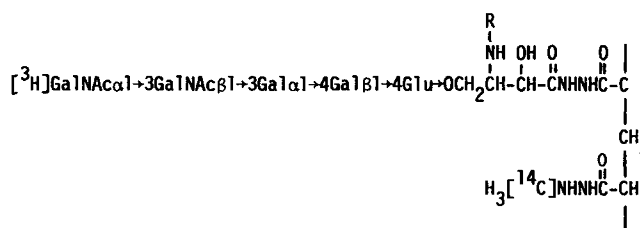

Fig. 2. Structure of Forssman-acid polyacrylic hydrazide polymer. *R* denotes the glycolipid fatty acid in amide linkage.

This water-soluble poly-glycolipid was also radiolabeled as follows. The Forssman glycolipid was labeled with ^3H using the galactose oxidase–$NaB[^3H_4]$ method (8) prior to coupling. In addition unreacted hydrazide groups were blocked with $[^{14}C]$-formaldehyde prior to the addition of unlabeled formaldehyde. The structure of this polyacrylic hydrazide polymer is shown in **Fig. 2.** Using this highly labeled, polymeric antigen, a sensitive radioimmunoassay has been developed for detecting Forssman glycolipid (13).

DISCUSSION

The method described in this paper for preparing solid supports or macromolecules that contain covalently linked glycolipids offers distinct advantages over alternative methods. The present method represents a simplified version of a previous procedure (1) and does not require ozone-generating equipment. For the affinity purification of anti-glycolipid antibodies, glycolipid acid–Sepharose columns are superior in terms of stability and capacity to columns prepared by the noncovalent trapping of glycolipid liposomes in acrylamide gels (14). Previous methods for coupling glycolipids to polymers involved removal of the *N*-acyl moiety with alkali followed by carbodiimide coupling or preparation of aminophenyl or

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aminobenzoyl compounds for diazotization (15, 16). However, these techniques were limited to the less complex glycolipids because of the sensitivity of the carbohydrate chains to alkaline degradation. The use of crown ether-solubilized potassium permangenate in benzene provides a gentle oxidant that can be used for both complex neutral glycolipids and gangliosides. The only restriction is that the sphingosine moiety must possess a susceptible structure; i.e., dihydrosphingosine-containing glycolipids are not suitable.

A number of current investigations in this laboratory utilize the specificity of solid supports or macromolecules bearing the carbohydrate determinants of glycolipids. The simplified method presented here for preparing these valuable reagents has aided in the progress of these studies.

This investigation was supported by the National Institutes of Health grant GM23100. W.W.Y. is the recipient of a fellowship from the Cancer Research Institute, Inc.

Manuscript received 9 May 1978; accepted 7 August 1978.

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