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Pre-packed reverse phase columns for isolation of complex lipids synthesized from radioactive precursors

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Summary Pre-packed reverse phase columns (Bond Elut) were used for the separation of complex lipids, such as phosphatidylcholine, cerebrosides, sulfatides, and gangliosides, from their respective water-soluble radioactive precursors after their in vitro biosynthesis. After an incubation in vitro, the entire reaction mixture is passed through the column, where complex lipids are retained and the hydrophilic radioactive precursors are washed away from the column. The retained lipids are then eluted with a more nonpolar organic solvent. The procedure is shown to be simpler and more efficient than the normally used Folch partitioning method or other procedures. —**Figlewicz, D. A., C. E. Nolan, I. N. Singh, and F. B. Jungalwala.** Pre-packed reverse phase columns for isolation of complex lipids synthesized from radioactive precursors. *J. Lipid Res.* 1985. **26:** 140–144.

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In order to measure the activity of various lipid-synthesizing enzymes in membranes and tissue fractions, in vitro incubations with water-soluble exogenous radioactive substrate are frequently used. Separation of the radioactive substrate from the products of the incubation is typically achieved using the classical

Abbreviations: SER, smooth endoplasmic reticulum; PAPS, phosphoadenosine 5'-phosphosulfate; CDP, cytidine diphosphate; CMP, cytidine monophosphate; UDP, uridine diphosphate; ATP, adenosine triphosphate; TUP, theoretical upper phase; HPLC, high performance liquid chromatography; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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phase-partitioning method of Folch, Lees, and Sloane Stanley (1). It was recently shown that use of prepacked C-18 reverse phase columns provided a rapid and effective method for isolating gangliosides from both small tissue samples and large scale preparations (2). Here we describe an application of 1.0-cc C-18 columns for separating the water-soluble radioactive precursor from the radioactive glycolipid or phospholipid products formed in the incubation mixtures. This method takes advantage of the difference in hydrophobic interaction of the labeled substrate and products to the C-18 bonded phase. When incubation mixtures are added to appropriately prepared C-18 columns, labeled lipid products formed are retained by the C-18 bonded phase, whereas the water-soluble precursor is removed through a series of aqueous washes. Subsequently, the lipid product is eluted with an organic solvent. This method serves as a convenient alternative to other separation methods.

MATERIALS AND METHODS

The following radioactive substrates were obtained from New England Nuclear (Boston, MA): phosphoadenosine 5'-phospho 3'-[^{35}S]-sulfate, (10.7 Ci/mmol); cytidine diphospho[methyl-^{14}C]-choline, (42.3 mCi/mmol); uridine diphosphate [U-^{14}C]galactose (302 mCi/mmol); and cytidine 5' monophosphate [4-^{14}C]- or [4,5,6,7,8,9-^{14}C]sialic acid, (1.8 mCi/mmol or 319 mCi/mol). Ultra-pure sucrose was obtained from International Biotechnologies, Inc. (New Haven, CT). Choline chloride was purchased from Eastman Kodak (Rochester, NY). Pre-packed Bond Elut, 1.0-cc C-18 columns and Vac Elut column holder were purchased from Analytichem International (Harbor City, CA). Egg yolk phosphatidylcholine, bovine brain phosphatidylethanolamine, and alpha-hydroxy fatty acid ceramides from bovine brain were obtained from Supelco (Bellefonte, PA), Avanti Polar Lipids, Inc. (Birmingham, AL), and Applied Sciences (State College, PA), respectively. Dithiothreitol, N-stearoyl dihydrolactocerebroside, uridine-5'-diphosphate-N-acetylglucosamine, Tween-20, adenosine triphosphate (disodium salt), galactocerebrosides, UDP galactose, and 1,2 diolein were obtained from Sigma (St. Louis, MO). Whatman No. 3 MM paper was obtained from Scientific Products. LKB-Ultrafilm for radioautography was purchased from LKB Instruments, Inc. (Gaithersburg, MD). All other reagents, ACS grade, were obtained from Fisher Scientific (Medford, MA).

Preparation of the enzyme sources

Brains from 18–20-day-old Sprague-Dawley rats (Charles River, Wilmington, MA) were used in preparing subcellular fractions. Microsomes were prepared from the cerebrum using the method of Cestelli, White, and Constantino-Ceccarini (3). Smooth endoplasmic reticulum (SER) was prepared from brainstem as described by Gilbert and Strocchi (4) except that heparin was omitted from Buffer A.

In vitro synthesis of galactocerebrosides, sulfatides, gangliosides, and phosphatidylcholine

UDP galactose:α-hydroxyacylsphingosine galactosyltransferase activity was studied in both microsomes and SER using the method of Cestelli et al. (3) with two modifications: egg phosphatidylcholine was substituted in the preparation of liposomes and 100 μg of bovine brain cerebrosides was added to each tube after termination of the enzyme reaction as carrier for newly synthesized cerebrosides. Synthesis of sulfatides was measured in SER as described by Jungalwala (5) for isolated subcellular particles. The synthesis of phosphatidylcholine was studied according to the incubation system of Jungalwala (6), except the amount of CDP [^{14}C]choline was decreased to 0.1 μCi per incubation tube. Sialyltransferase activity in SER for the synthesis of GM_3 was assayed by the method of Basu et al. (7). For all samples, duplicate blanks were prepared by incubating the reaction mixture without the enzyme source and the appropriate amount of the enzyme was added after termination of the reaction.

Folch extraction

In the case of samples processed using the method of Folch et al. (1), the enzyme reactions were terminated by addition of 10.0 ml of chloroform–methanol 2:1 (v/v). The chloroform–methanol extract was washed with 0.2 vol of 0.9% NaCl and the upper phase was removed. The lower phase was then washed three times with theoretical upper phase (TUP) containing a carrier: either 1.0 mM galactose, 10.0 mM sodium sulfate, or 7.5 mM choline chloride for the in vitro assay of cerebrosides, sulfatides, or phosphatidylcholine synthesis, respectively, followed by three washes with TUP without the respective carrier (1).

Paper chromatography

Sialyltransferase reaction for the synthesis of GM_3 was terminated by the addition of 0.25 ml of chloroform–methanol 1:1. The entire incubation mixture was spotted on Whatman No. 3 MM paper and chromatographed in a descending direction as described by Chien, Williams, and Basu (8). The unreacted radioactive nucleotide sugar precursors migrated with the solvent front, whereas the major product GM_3 ganglioside remained at the origin. The ganglioside product at the origin was localized by radioautography; the paper

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with the radioactive product was cut and placed in a scintillation vial.

Separation using reverse phase C-18 columns

In case of samples processed with C-18 reverse phase columns, the enzyme reactions were terminated by addition of 5.0 ml of TUP prepared with 0.1M KCl instead of saline. Up to ten samples can be processed simultaneously when Bond Elut C-18 columns are placed in the Vac Elut column holder. All column procedures were carried out using constant gentle vacuum. Prior to loading of the incubation mixture on the columns, the columns were washed successively with 5.0 ml of chloroform–methanol 2:1 (v/v); 5.0 ml of distilled water, and again with 5.0 ml of chloroform–methanol 2:1. The C-18 columns were then equilibrated with 5.0 ml of TUP which contained either 1.0 mM galactose, 10 mM sodium sulfate, or 7.5 mM choline chloride as a carrier as previously described. Appropriate incubation mixtures were then passed through the column followed by washes with 5.0 ml of TUP and 10.0 ml of distilled water. Each time the original reaction mixture tube was rinsed with the solvent before loading onto the column to assure complete transfer. The tubes were then rinsed with 7.0 ml of chloroform–methanol 2:1 and the solvent passed through the column to elute cerebrosides, sulfatides, or phosphatidylcholine. The lipids eluted from the column with the last solvent were collected directly into scintillation vials and the solvent was evaporated.

In the case of ganglioside synthesis in vitro, the procedure is similar to that described by Williams and McCluer (22). The reaction was terminated with 2 ml of TUP and the mixture was loaded onto the prewashed and equilibrated column as described above. The reaction tube was rinsed three times with 1 ml each of TUP and loaded onto the column. The combined eluate was recycled twice to ensure complete binding of the gangliosides. The column was then washed with 25 ml of distilled water. The gangliosides were eluted with 3 ml of methanol, collected directly in a scintillation vial, and the solvent was evaporated.

Determination of radioactivity

All radioactive products were redissolved or suspended in Scinti-Verse I scintillant and counted in a Packard scintillation counter.

RESULTS AND DISCUSSION

Four different classes of lipids, viz, cerebrosides, sulfatides, phosphatidylcholine, and gangliosides, were synthesized in vitro under conditions that included exogenous radioactive substrates. Results were analyzed by the Bond Elut method and compared with the normally used method of Folch et al. (1) or Chien et al. (8) (**Tables 1–4**). With either of the methods of lipid isolation, linearly increasing amounts of various radioactive products were obtained with increasing amounts of the enzyme source (microsomes or SER) in the range of 10–500 μg of protein. The blank values for the Bond Elut method were similar to blanks obtained by other methods, indicating that the water-soluble precursor was almost completely washed away from the Bond Elut column without the loss of the lipid products. In the case of the sialyltransferase assay, the blank values were high by both methods used (Table 4) and appeared to vary with the amount of enzyme. In more recent experiments, extensive aqueous washing (up to 50 ml) of the Bond Elut column reduced the blank values to about 200 cpm with 200 μg of protein in the assay. Addition of 1.0 mM N-acetylglucosamine carrier to the TUP or an additional wash with 10% methanol in water did not improve this latter result. The correlation coefficient, *r*, (determined by linear regression analysis) between the contrasted methods, shown at the bottom of each table, was excellent. Overall results show that for the isolation of lipids after in vitro enzyme reactions, the Bond Elut method was as reliable as the other two methods compared. The new method is comparatively more efficient when a large number of enzymatic assays of the type reported here are to be carried out at the same time. In our experience, ten samples can be processed in about an hour compared to several hours or longer for the other two procedures. We have used the new procedure for rapid enzyme assay of fractions obtained during enzyme

TABLE 1. Incorporation of [^{35}S]phosphoadenosine 5'-phosphosulfate into sulfatides

	Bond Elut Method	Folch Method
	cpm	
SER protein (μg)		
10	60 ± 12	53 ± 3
20	141 ± 11	130 ± 21
50	799 ± 99	718 ± 149
100	1950 ± 303	1790 ± 126
200	5240 ± 365	5410 ± 1020

Correlation coefficient from linear regression analysis, *r*, between the two methods = 0.999

Values are average of triplicate determinations ± standard error of the mean and listed as net cpm. The blank values averaged 105 cpm for the Bond Elut method and 42 cpm for the Folch method. Each assay tube contained bovine brain cerebrosides, 80 μg; ^{35}S-labeled PAPS, 0.25 μCi (15 ng); 0.2% Triton; 100 mM Tris, pH 7.15; 2.5 mM ATP, 19.5 mM $MgCl_2$; and varying amounts of SER protein as indicated above in a total volume of 0.5 ml. Samples were incubated for 1 hr at 37°C with gentle shaking.

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TABLE 2. Incorporation of CDP-[^{14}C]choline into phosphatidylcholine

	Bond Elut Method	Folch Method
	cpm	
SER protein (μg)		
5	866 ± 43	1270 ± 245
10	1780 ± 226	2070 ± 220
20	4600 ± 293	3620 ± 259
50	11500 ± 705	11700 ± 1320
100	32600 ± 1280	35300 ± 2660
200	68700 ± 1680	76800 ± 3630
500	159000 ± 6530	167000 ± 3370

Correlation coefficient from linear regression analysis, *r*, between the two methods = 0.99

Values are average of triplicate determinations ± standard error of the mean and listed as net cpm. The blank values averaged 145 cpm for the Bond Elut method and 122 cpm for the Folch method. Varying amounts of SER protein (as indicated above) were incubated with CDP-[^{14}C]choline, 0.1 μCi (2.4 nmol); 250 μg diolein; 36 mM $MgCl_2$; 50 mM Tris-HCl, pH 7.4; and 0.02% Tween-20 in a total volume of 750 μl, for 1 hr at 37°C.

purification, and for assays in different subcellular fractions, in samples from tissue culture, and in different tissues during development (9). In addition to the enzyme assays reported here, we have also successfully used the Bond Eult procedure for the assay of other lipid synthesizing enzymes. In vitro activities of CDP-diacylglycerol:myo-inositol 3-phosphatidyltransferase (synthesis of phosphatidylinositol), CDP-ethanolamine: 1,2 diacylglycerol ethanolaminephosphotransferase (synthesis of phosphatidylethanolamine), and UDP-glucose: ceramide glucosyltransferase (synthesis of glucocerebroside), have been determined using [^{3}H]inositol, CDP-[^{14}C]ethanolamine, and UDP-[^{14}C]glucose as respective radioactive precursors. The Bond Elut method was also successfully used for the separation of derivatized lipids, such as perbenzoylated cerebrosides and monosialogangliosides (used in HPLC), from the hydrophilic reagent benzoylchloride and its side products. The HPLC profile of perbenzoylated cerebrosides and gangliosides isolated by the Bond Elut method was identical to that obtained by previous methods (10–12). A similar use of C-18 reverse phase columns to adsorb gangliosides from aqueous solutions has been previously described (2). The present method will prove applicable to other experimental systems as well, where polar molecules are to be resolved from the relatively nonpolar compounds.

TABLE 3. Incorporation of UDP-[^{14}C]galactose into cerebrosides

	Bond Elut Method	Folch Method
	cpm	
Protein (μg)		
Microsomal		
14	510; 477	491; 309
28	897; 880	810; 974
42	1114; 1241	1345; 1134
SER		
10	905 ± 73	830 ± 47
20	1330 ± 5	1054 ± 4
30	1493 ± 12	1369 ± 44
50	2033 ± 110	1954 ± 63

Correlation coefficient from linear regression analysis, *r*, between the two methods = 0.97

In the case of SER, values are average of triplicate determinations ± standard error of the mean, listed as net cpm after subtracting the blank values. The blank values averaged 86 cpm for the Bond Elut method and 79 cpm for the Folch method. Each reaction mixture contained 40 μl of liposome substrate with 76 μg of ceramide, 500 μg of phosphatidylethanolamine, and 50 μg of phosphatidylcholine (3), UDP-[^{14}C]galactose 0.05 μCi, 164 μg of unlabeled UDP-galactose, 50 mM Tris-HCl (pH 9.0), 1.0 mM DTT, 1.0 mM EDTA, 10.0 mM $MgCl_2$, and varying amounts of enzyme protein (as indicated above) in a total volume of 150 μl. Samples were incubated for 1 hr at 37°C with gentle shaking.

TABLE 4. Incorporation of CMP-[^{14}C]-NANA into gangliosides

	Bond Elut Method	Chromatography
	cpm	
SER protein (μg)		
20	301 ± 64	368 ± 40
50	1240 ± 129	1310 ± 50
100	2710 ± 204	3020 ± 43
200	5070 ± 132	6210 ± 384

Correlation coefficient from linear regression analysis, *r*, between the two methods = 0.997

Values are average of triplicate determinations ± standard error of the mean and listed as net cpm. The blank values averaged 550 cpm for the Bond Elut method and 302 cpm for the paper chromatography method. Each assay mixture contained 0.5 mM ceramide lactoside, 200 mM cacodylate-HCl buffer (pH 6.4), 2.5 mM $MgCl_2$, 0.2% Triton CF-54, 0.5 mM EDTA, 0.232 mM CMP-[^{14}C]-NANA (3.4 × 104 dpm/n mol) and varying amounts of enzyme protein as indicated above, in a total volume of 0.05 ml. The incubation was for 1.5 hr at 37°C.

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