Retention of gangliosides in serum delipidated by diisopropyl ether–1-butanol extraction

Clifford G. Wong and Stephan Ladisch[1]

Division of Hematology/Oncology and Gwynne Hazen Cherry Memorial Laboratories, Department of Pediatrics, UCLA School of Medicine, Los Angeles, CA 90024

Summary Extraction with diisopropyl ether–1-butanol is a rapid method for the delipidation of serum without protein denaturation. We sought to confirm that this solvent system would also extract the highly polar acidic glycosphingolipids, gangliosides. In fact, however, both endogenous serum gangliosides and radiolabeled rat brain gangliosides added to serum were nearly quantitatively retained (87.5% and 98.7%, respectively) in the aqueous phase after two extractions. Therefore, while useful for the extraction of most lipids, this delipidation procedure cannot be used to remove gangliosides from serum or plasma.—**Wong, C. G., and S. Ladisch.** Retention of gangliosides in serum delipidated by diisopropyl ether–1-butanol extraction. *J. Lipid Res.* 1983. **24:** 666–669

Supplementary key words serum gangliosides • rat brain gangliosides • phospholipids

Cham and Knowles (2) recently developed a biphasic solvent system using diisopropyl ether (DIPE)–1-butanol to simply, rapidly, and completely delipidate serum or plasma. The major advantage of this method over others, such as that of Folch, Lees, and Sloane Stanley (3), is that serum proteins are not precipitated or denatured, thereby allowing complete recovery of enzyme activities and protein antigenicity in the delipidated sample (2).

The quantitative extraction of triglycerides, cholesterol, free fatty acids, and phospholipids by the procedure of Cham and Knowles has been documented (2, 4). We sought to confirm that, as also reported (5), this procedure removes the more polar sialic acid-containing glycosphingolipids (gangliosides), allowing use of the method for the quantitative extraction of gangliosides from serum. Our findings indicate, however, that both endogenous serum gangliosides and rat brain gangliosides (added to serum) are not significantly extracted into the organic phase by this procedure, but rather are nearly quantitatively retained in the aqueous phase.

Abbreviations: DIPE, diisopropyl ether; TLC, thin-layer chromatography; LBSA, lipid-bound sialic acid; ganglioside nomenclature according to Svennerholm (1).

[1] To whom reprint requests should be addressed.

MATERIALS AND METHODS

All solvents used were reagent grade (Mallinckrodt, St. Louis, MO). [1-^{14}C]-*N*-acetyl-D-mannosamine (18 mCi/mmol) was obtained from ICN (Irvine, CA) and [1-^{14}C]dipalmitoylphosphatidylcholine (100 mCi/mmol) was from New England Nuclear (Boston, MA). Rat brain gangliosides were radiolabeled in vivo with [1-^{14}C]-*N*-acetyl-D-mannosamine, which is incorporated into the sialic acid moiety of gangliosides (6). Thirty μCi of the label, dissolved in isotonic salt solution, was injected intracerebrally into 15-day-old Sprague-Dawley rats that were killed 12 hr later. The brains were removed and lyophilized. The gangliosides were extracted with chloroform–methanol and purified by silicic acid chromatography (7) followed by Sephadex G-50 (Pharmacia, Uppsala, Sweden) gel-filtration to remove unincorporated label, nucleotide sugars, and other water-soluble, low molecular weight contaminants (8). Mixed bovine brain gangliosides (Supelco, Bellefonte, PA) were similarly purified.

Gangliosides were quantitated as nmol of lipid-bound sialic acid (LBSA) by the modified resorcinol colorimetric assay (9). Thin-layer chromatography was performed using 10 × 10 cm high-performance TLC silica gel 60 plates (E. Merck, Darmstadt, West Germany) developed in chloroform–methanol–0.25% $CaCl_2 .2H_2O$ 60:40:9 (v/v/v) (9). Gangliosides were visualized with resorcinol-HCl reagent (9). Radioautography of the rat brain gangliosides was performed by exposing the TLC plate to Kodak (Rochester, NY) XRP-5 X-ray film at −80°C.

Human serum was delipidated with DIPE–1-butanol as described (2), with minor modifications suggested to minimize protein emulsification at the organic-aqueous phase interface.[2] One volume of serum was mixed with two volumes of DIPE–1-butanol 60:40 (v/v) in glass centrifuge tubes with Teflon-lined caps on a rocking-bed agitator (Bellco Glass, Inc., Vineland, NJ) for 1 hr at a rocking rate of 10 rpm. The tubes were then allowed to stand for 5 min before centrifugation (150 *g* for 5 min) to separate the phases. The upper organic phase was removed and two volumes of diethylether were added to the aqueous phase. The tube was carefully inverted five times by hand, and centrifuged as above. The clear ether layer was removed and added to the organic phase. The extraction procedure was repeated on the aqueous phase, and the organic phases were pooled. Both phases were taken to dryness by rotary evaporation and lyophilization. The organic phase residue was redissolved in chloroform–methanol 1:1,

[2] Cham, B. E. Personal communication.

and gangliosides were separated from neutral lipids by silicic acid chromatography (10) and quantitated. Gangliosides retained in the aqueous phase were isolated by two extractions of the aqueous phase residue with chloroform–methanol 1:1 (v/v). This lipid extract was treated by alkaline methanolysis (9) to remove phospholipids, redissolved in distilled water with brief sonication in a Branson (Shelton, CT) water bath sonicator, and desalted by Sephadex G-50 gel filtration (8). These gangliosides were then lyophilized, dissolved in chloroform–methanol 1:1 (v/v), quantitated, and qualitatively characterized by TLC.

In the other experiments 9500 cpm of radiolabeled rat brain gangliosides was added to 2.5 ml of serum or saline, aliquots were removed to determine total radioactivity, and DIPE–1-butanol extraction was then performed twice on the remaining 2.0 ml, exactly as above. The organic phases were pooled, dried under nitrogen, and solubilized in 1.5 ml of Soluene-350 (Packard, Burlingame, CA) for 18 hr. An aliquot of the twice-extracted final aqueous phase was treated in the same manner. Fifteen ml of Aquasol-2 (New England Nuclear, Boston, MA) was then added to each sample. The samples were dark-adapted and counted on a Beckman Model LS-8000 β-scintillation counter, and total radioactivity in each phase was calculated.

The extraction from serum and saline of a radiolabeled phospholipid, [1-^{14}C]dipalmitolyphosphatidylcholine, was measured in an identical manner using approximately 30,000 cpm (0.2 nmol) per experiment.

RESULTS

The distribution of endogenous gangliosides in the aqueous and organic phases following two extractions of serum with DIPE–1-butanol is shown in **Table 1.** Most (87.5%) of the total gangliosides were retained in the aqueous phase. The total serum ganglioside concentrations, 14.2–14.9 nmol LBSA/ml, were within the range of normal mean values (11–20 nmol LBSA/ml) previously reported (11–13). TLC of the gangliosides recovered in the aqueous phase (**Fig. 1**) demonstrated both G_{M3} and structurally more complex gangliosides to be present. Densitometric scanning of the same TLC showed G_{M3} to constitute 76%, and structurally more complex gangliosides, 24%, of the total gangliosides recovered in the aqueous phase.

The retention of gangliosides in the aqueous phase was confirmed in other experiments using radiolabeled gangliosides obtained from rat brain. Purity of these gangliosides was assessed by TLC, in which radioautography and resorcinol-HCl staining of the same lane (**Fig. 2**) revealed almost identical patterns of bands, indicating

TABLE 1. Partition of gangliosides and phospholipid by DIPE–1-butanol extraction

Experiment	Total	Final Aqueous Phase[a]	Combined Organic Phases[a]	% Extracted into Organic Phase
Serum gangliosides (nmol LBSA/ml serum)[b]				
1	14.2	12.4	1.8	12.7
2	14.9	12.8	2.1	14.1
3	14.9	13.3	1.6	10.7
				12.5 ± 1.7[c]
^{14}C-labeled rat brain gangliosides (cpm × 10^{-3}/sample)[d]				
4	7.24	7.29	0.09	1.2
5	7.74	7.49	0.09	1.2
6	7.57	7.55	0.12	1.5
7	7.62	7.77	0.10	1.3
				1.3 ± 0.1[c]
8	7.99	7.71	0.02	0.3
[^{14}C]dipalmitoylphosphatidylcholine (cpm × 10^{-3}/sample)[e]				
9	22.4	3.2	20.2	86.4
10	24.5	3.5	20.5	85.5
11	22.8	3.9	19.6	83.4
12	27.4	4.1	24.6	85.6
13	27.9	2.3	20.4	89.9
				86.2 ± 2.4[c]
14	24.0	0.0	25.2	100
15	20.8	0.2	21.6	99.0
16	19.9	0.1	21.2	99.7
				99.6 ± 0.5[c]

[a] After two extractions.
[b] Different 10-ml samples (Expts. 1–3) were extracted and the ganglioside content in each phase was quantitated.
[c] Mean ± SD.
[d] Two-ml samples of serum (Expts. 4–7) or 0.9% saline (Expt. 8) to which radiolabeled rat brain gangliosides had been added were extracted and radioactivity in each phase was quantitated.
[e] Two-ml samples of serum (Expts. 9–13) or 0.9% saline (Expts. 14–16) to which [^{14}C]dipalmitoylphosphatidylcholine had been added were extracted and the radioactivity in each phase was quantitated.

that the purified rat brain ganglioside preparation was essentially free of radiolabeled non-ganglioside contaminants and unincorporated label. The TLC (Fig. 2) also shows only gangliosides structurally more complex than G_{M3} to be present in our rat brain preparation, as compared to serum, in which G_{M3} is the predominant ganglioside (Fig. 1). Specific activity of the purified gangliosides was 223 cpm/nmol LBSA, and G_{D1a} and G_{T1b} were the most heavily labeled (Fig. 2).

Extraction of these radiolabeled rat brain gangliosides from serum by DIPE–1-butanol was minimal (Table 1); greater than 98% of the radioactivity was retained in the aqueous phase. Radiolabeled gangliosides added to saline were likewise retained in the aqueous phase (Table 1), excluding the possibility that the retention of gangliosides in the aqueous phase during serum extraction could have been due to protein-lipid

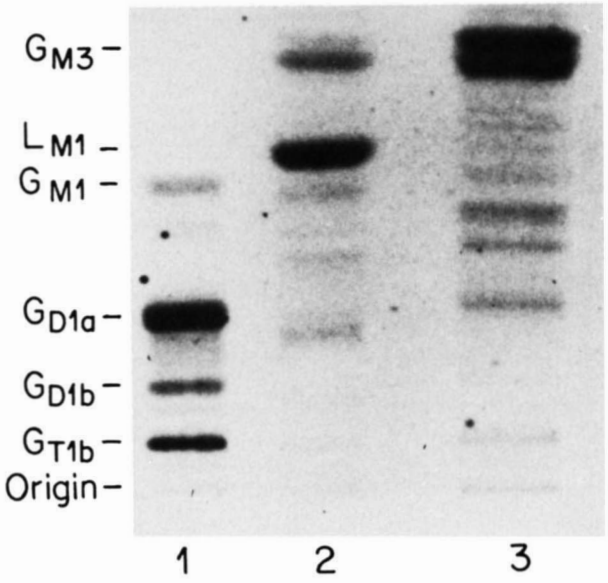

Fig. 1. Gangliosides retained in the aqueous phase after DIPE–1-butanol extraction of serum. Normal human serum was extracted twice with DIPE–1-butanol. Gangliosides in the final aqueous phase were isolated and purified (see Methods) and chromatographed. Lane 1: mixed bovine brain ganglioside standards, 10 nmol LBSA; lane 2: mixed human erythrocyte ganglioside standards; 4 nmol LBSA; lane 3: serum gangliosides retained in the aqueous phase, 10 nmol LBSA. All bands above the origin are resorcinol-positive.

interactions or could have reflected hydrolytic release of ganglioside [^{14}C]sialic acid by serum sialidases, with subsequent retention of the free [^{14}C]sialic acid in the aqueous phase.

Parallel studies confirmed the efficiency of phospholipid extraction by the method of Cham and Knowles under the experimental conditions described in Methods. More than 86% and 99% of added [1-^{14}C]dipalmitoylphosphatidylcholine was removed from serum and saline, respectively, by two extractions with DIPE–1-butanol (Table 1), in agreement with the previously reported nearly quantitative extraction of phospholipid by this method (2, 4, 5). These results excluded the possibility that the retention of gangliosides in the aqueous phase in our experiments simply reflected poor extraction of lipids other than gangliosides.

DISCUSSION

In this study, the partitioning of endogenous serum gangliosides in the two phases of the delipidation procedure of Cham and Knowles (2) was determined. Retention of serum gangliosides in the aqueous phase was nearly complete, with only 12.5% of the total gangliosides being extracted into the organic phase. The thin-layer chromatogram of gangliosides isolated and purified from the final aqueous phase, and densitometric analysis of this TLC, demonstrated preservation of the reported qualitative distribution of total serum gangliosides (66–88% G_{M3}) using standard ganglioside isolation methods (11, 12), further supporting the conclusion that gangliosides in serum are not extracted with other lipids by DIPE–1-butanol.

Gangliosides structurally more complex and more hydrophilic than G_{M3}, and having TLC mobilities in the same range of those of the major brain gangliosides (G_{M1} through G_{Q1b}), comprise only a small (12–34%) fraction of all serum gangliosides (11, 12). To more accurately establish the fate of such complex gangliosides in this delipidation procedure we therefore measured the extraction, from serum, of brain gangliosides added to serum. Together the results (Table 1) allow the conclusion that both the predominant serum ganglioside, G_{M3}, and the minor, more complex species, are nearly quantitatively retained in the aqueous phase.

Our results contrast with the report of Albouz et al. (5), that plasma gangliosides are extracted into the organic phase. We cannot explain this difference. Our extraction method was not different and we found that neither the extraction of plasma instead of serum nor the presence of EDTA in the sample increased the extraction of gangliosides (not shown). Albouz et al. (5) did state that slight modifications of the procedure of Cham and Knowles (2) were made, but since these were not described in the text (5), whether they could have caused gangliosides to be extracted from the aqueous phase cannot be determined.

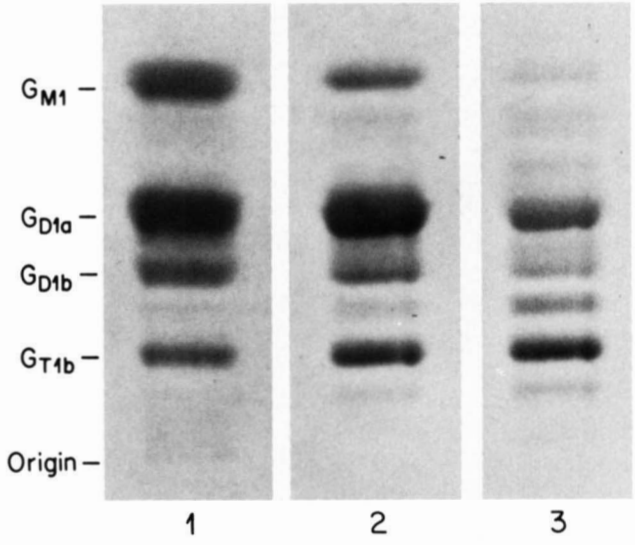

Fig. 2. Characterization of radiolabeled rat brain gangliosides. The TLC pattern of ^{14}C-labeled rat brain gangliosides (10 nmol LBSA) visualized by resorcinol-HCl staining (lane 2) is compared with that of the same lane detected by radioautography (lane 3). Lane 1: bovine brain gangliosides (10 nmol LBSA), resorcinol stain. All bands in lanes 1 and 2 are resorcinol-positive.

On the other hand, the original data of Cham and Knowles (2) suggest an explanation for our findings of almost quantitative retention of gangliosides in the aqueous phase. They found that the most polar of the lipids they studied (i.e., phospholipids) were the least readily extracted. The even more polar and hydrophilic characteristics of gangliosides could therefore be responsible for the retention of these glycosphingolipids in the aqueous phase of serum extracted with DIPE–1-butanol.

We thank R. Dollinger and Drs. B. Gillard and R. Shearer for assistance in ganglioside radiolabeling and TLC, and D. Ross for preparation of the manuscript. This work was supported by Grant CA 27701 from the National Cancer Institute and aided by Clinical Research Grant 6-268 from the March of Dimes Birth Defects Foundation. Dr. Ladisch is the recipient of Research Career Development Award CA 00821 from the National Cancer Institute and is a Scholar of the Leukemia Society of America.

Manuscript received 29 March 1982 and in revised form 13 January 1983.

REFERENCES

1. Svennerholm, L. 1963. Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10:** 613–623.
2. Cham, B. E., and B. R. Knowles. 1976. A solvent system for delipidation of plasma or serum without protein precipitation. *J. Lipid Res.* **17:** 176–181.
3. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
4. Blumenfeld, O. O., E. Schwartz, and A. M. Adamany. 1979. Efflux of phospholipids from cultured aortic smooth muscle cells. *J. Biol. Chem.* **254:** 7183–7190.
5. Albouz, S., J. Galli, R. Bourdon, and N. Baumann. 1979. Method d'extraction des lipides plasmatiques conservant les proprietes antigeniques de proteines et permettant le dosage de l'acide sialique des gangliosides. *Ann. Biol. Clin.* **37:** 287–290.
6. Quarles, R. H., and R. O. Brady. 1971. Synthesis of glycoproteins and gangliosides in developing rat brain. *J. Neurochem.* **18:** 1809–1820.
7. Irwin, C. C., and L. N. Irwin. 1979. A simple rapid method for ganglioside isolation from small amounts of tissue. *Anal. Biochem.* **94:** 335–339.
8. Ueno, K., S. Ando, and R. K. Yu. 1978. Gangliosides of human, cat, and rabbit spinal cords and cord myelin. *J. Lipid Res.* **19:** 863–871.
9. Ledeen, R. W., and R. K. Yu. 1982. Gangliosides: structure, isolation, and analysis. *Methods Enzymol.* **83:** 139–191.
10. Saito, M., and A. Rosenberg. 1982. Glycolipids and their development patterns in chick thigh and leg muscles. *J. Lipid Res.* **23:** 3–8.
11. Yu, R. K., and R. W. Ledeen. 1972. Gangliosides of human, bovine, and rabbit plasma. *J. Lipid Res.* **13:** 680–685.
12. Portoukalian, J., G. Swingelstein, N. Abdul-Malak, and J. F. Doré. 1978. Alteration of gangliosides in plasma and red cells of humans bearing melanoma tumors. *Biochem. Biophys. Res. Commun.* **85:** 916–920.
13. Kloppel, T. M., TW. Keenan, M. J. Freeman, and D. J. Morré. 1977. Glycolipid-bound sialic acid in serum: increased levels in mice and humans bearing mammary carcinomas. *Proc. Natl. Acad. Sci. USA.* **74:** 3011–3013.