methods

Chromatographic resolution and quantitative assay of CNS tissue sphingoids and sphingolipids

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Abstract **Quantitative separation of ceramide, sphingoids (dihydrosphingosine, sphingosine, psychosine), and glycosphingolipids as individual fractions was achieved with silicic acid, Dowex column chromatography, and specific solvent mixtures that have not been previously described. Purified ceramide, resolved as a single band, was assayed by thin-layer chromatography (TLC) followed by gas chromatography (GC) and high performance liquid chromatography (HPLC). Sphingoids, purified by Dowex, were assayed by GC and HPLC without mild alkaline hydrolysis, which reduces the yield by interfering with the free amino group of psychosine and dihydrosphingosine. Several less polar (than cerebroside) alkali-/acid-labile glycosphingolipids that elute with galactosylceramide were also identified. Neutral and acidic glycosphingolipids, quantitatively recovered and purified to homogeneity, were resolved by TLC.** We used these techniques to determine sphingolipids and sphingoids of vertebrate central nervous system (CNS) tissue, using as little as 30–50 mg (wet weight) of tissue. In addition, phosphatidylcholine and sphingomyelin, relevant to ceramide metabolism, were quantitatively recovered in pure form and resolved by TLC. This method, used to study CNS sphingolipid content, may well be applicable to determine the sphingolipid composition of other tissues and cell culture, but further experiments are necessary to ascertain this.—Dasgupta, S., and E. L. Hogan. **Chromatographic resolution and quantitative assay of CNS tissue sphingoids and sphingolipids.** ***J. Lipid Res.*** **2001.** 42: **301–308.**

Supplementary key words ceramide • chromatography • ganglioside • neutral glycosphingolipid • phospholipid • sphingomyelin

Sphingolipids contain a core structure of ceramide, a fatty acid attached to the C-2 amino group of the aliphatic long-chain sphingosine (or dihydro- or phytosphingosine). Ceramide and other sphingolipid metabolites participate in a wide variety of biological phenomena (1). For example, ceramide modulates protein phosphorylation, the activity of protein kinase C (PKC), and phospholipase A_2, and mediates signal transduction (2, 3), while sphingosine and psychosine are potent inhibitors of PKC (4). Specific glycosphingolipids (GSL)[2] are receptors for bacterial toxins, modulators of transmembrane signal inducers, and mediators of cell-cell recognition, cell growth, and differentiation (5). In addition to de novo synthesis, hydrolysis of sphingomyelin (SM) by sphingomyelinase is an alternative source of in vivo ceramide generation (3). Biological functions of sphingoids and sphingolipids in tissues and cells are pertinent to their relative concentrations and, hence, their precise quantitation is of great importance. Although a variety of methodologies are used for assaying individual components, there is no satisfactory systematic analytical procedure that is suitable for complete examination and quantitative assay of these membrane molecules and their metabolites. Most published methods examine either a particular lipid or a lipid group such as ceramide, GSL, phospholipid, or sphingoid after specific chemical treatments (e.g., alkaline methanolysis) that disrupt specific linkages of certain lipids (6–8). Such methods are suitable only for a large batch preparation and may not be applicable for examination of total lipids, using a small amount of tissue or cell culture (9).

We have developed a method that can resolve and assay sphingolipid and sphingoid contents in a small quantity of (CNS) central nervous system tissue, using sequential silicic acid and Dowex column chromatography, eluted with various compositions of solvents. This chromatographic resolution and quantitation (CRAQ) may be the first systematic quantitative approach for examining in vivo sphingoid and sphingolipid composition and should facilitate more accurate assessment of sphingolipid metabolism in vitro.

MATERIALS AND METHODS

Dowex 50WX8-200, silicic acid, and phospholipids standards, that is, phosphatidylcholine (PC), phosphatidylethanolamine

[2] Ganglioside and glycosphingolipid short designations are according to L. Svennerholm (*J. Neurochem.* 1963;**10:**613–623) and the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Lipid Res.* 1978; **19:** 114–128).

Abbreviations: CNS, central nervous system; DAG, diacylglycerol; DIG-IS, digoxigenin immunostaining; GC, gas chromatography; GSL, glycosphingolipid; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; MGC, monoglycosylceramide; NGSL, neutral glycosphingolipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; THF, tetrahydrofuran; TLC, thin-layer chromatography.

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(PE), phosphatidylserine (PS), and phosphatidylinositol (PI), were purchased from Sigma-Aldrich (St. Louis, MO); Florisil and precoated thin-layer chromatography (TLC) plates were from EM Science (Gibbstown, NJ); Sep-Pak C18 cartridges were from Waters (Milford, MA); diethylaminoethyl (DEAE)-Sephadex A50 was from Pharmacia LKB Biotechnology (Uppsala, Sweden). Digoxigenin and anti-digoxigenin antibody (Fab fragments) were obtained from Roche Diagnostics (Indianapolis, IN). Standard neutral glycosphingolipids (NGSL) and SM were purified from bovine erythrocytes (10) and gangliosides were purified from bovine brain and characterized in our laboratory (11). Solvents and other reagents were of analytical grade. Fresh bovine brain and spinal cord were collected from a local abattoir and frozen at −40°C until use.

White matter and gray matter were carefully dissected from frozen brain. Tissue (30–50 mg) was homogenized with chloroform–methanol–water 2:4:1 (v/v/v; 14 ml/g of tissue) (10), stirred for 1 h and centrifuged; the pellet was re-extracted with the same solvent. A third extraction was carried out with chloroform–methanol 2:1 (v/v). Purification and characterization of sphingolipids and sphingoids, shown diagrammatically in **Fig. 1**, are described below.

Purification of ceramide, MGCs, GSLs, and phospholipids

The three extracts were pooled, dried, and suspended in a minimum volume of chloroform. Sphingolipids [e.g., ceramide, monoglycosylceramides (MGC), GSL] were purified as individual fractions as described below.

The chloroform suspension was applied to a silicic acid column (0.5 × 5 cm) and washed with 15 column volumes of chloroform to remove nonpolar lipids (which can be assayed by TLC). The column was then eluted successively with *1*) chloroform–acetone 9:1 (v/v; 15 column volumes; for ceramide), *2*) chloroform–methanol 23:2 (v/v; 10 column volumes; for MGC), *3*) tetrahydrofuran (THF)–water 7:1 (v/v; 3.5 column volumes; for long-chain GSL, sphingoids, and some phospholipids, e.g., cardiolipin, PE, PI, PS; phospholipid fraction 1), and *4*) methanol (5 column volumes, phospholipid fraction 2, for PC and SM). Each fraction was dried and stored at 4°C until use.

Examination and assay of ceramide

Ceramide, purified by silicic acid chromatography [chloroform–acetone 9:1 (v/v)], was dissolved in a defined volume of chloroform (1.0 ml/g tissue) and 15 μl was applied to a high performance thin-layer chromatography (HPTLC) plate, developed with chloroform–methanol–acetic acid 95:4.5:0.5 (v/v/v) (12) and visualized by benzidine spray (13). A measured volume (5 μl) of the ceramide solution was removed and methanolyzed with methanol–water–HCl 29:4:3 (v/v/v) (14) [most effective of several available methods (15–18)] at 80°C for 18 h, and the recovered base was analyzed by gas chromatography (GC) as the trimethylsilyl derivative (19). Ceramide was also assayed by high performance liquid chromatography (HPLC) as the 3-keto derivative (20). The recovery of the measured standard ceramide purified through the silicic acid column, using chloroform–acetone 9:1 (v/v), was greater than 95%.

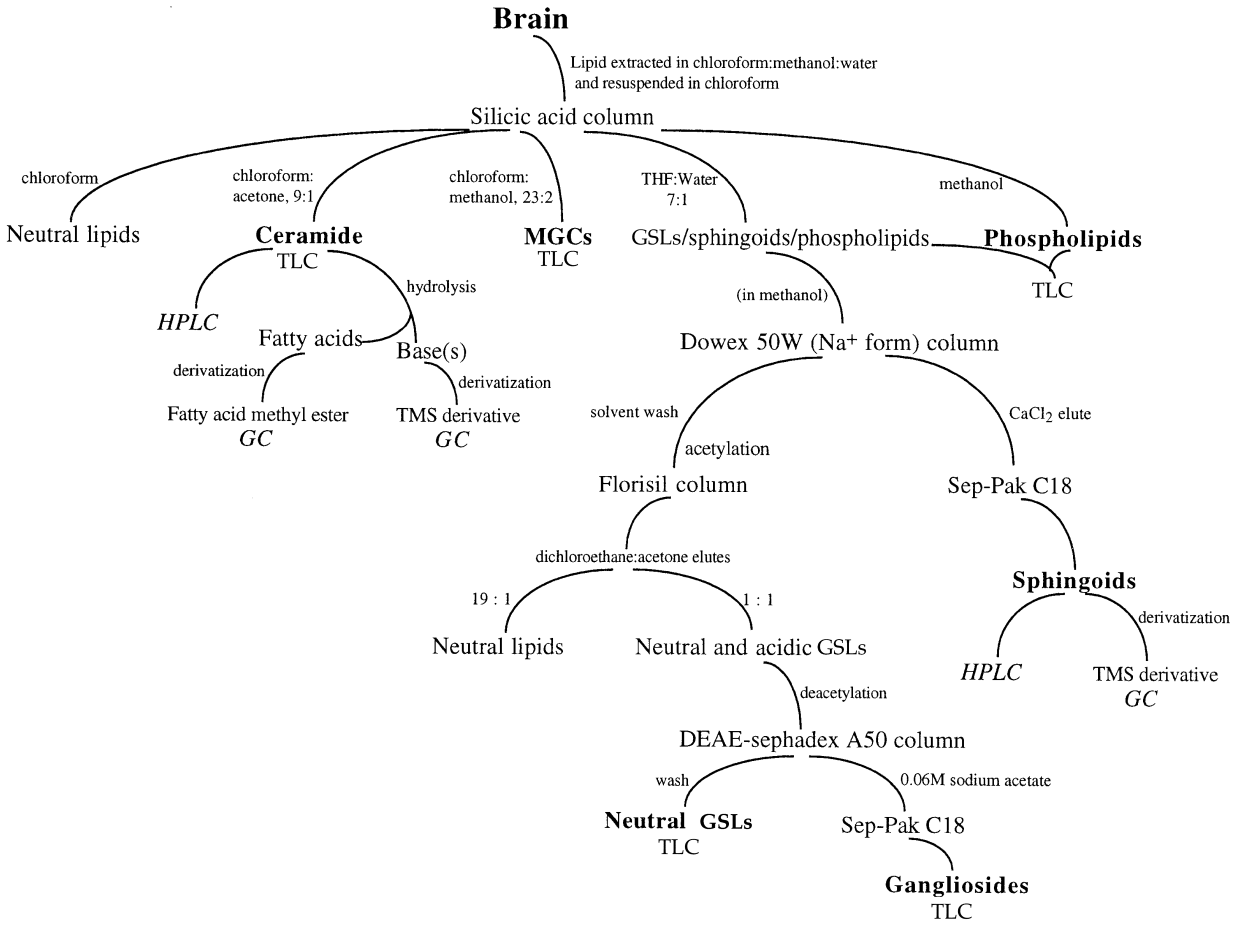

Fig. 1. Diagrammatic presentation of the stepwise separation of bovine CNS lipids by column chromatography. TMS, trimethylsilyl.

Purification and assay of sphingoids

The THF-water elution was dissolved in chloroform–methanol–water 12:7:1 (v/v/v; 15 ml/g tissue), 50 μl was preserved (for examining phospholipids), and the rest was dried and resuspended in methanol. Sphingoids were separated by a Dowex 50WX8-200 column (1 × 2 cm; Na^+ form) previously equilibrated with methanol (21). After washing with 15 column volumes of *1)* methanol, *2)* chloroform–methanol 2:1 (v/v), and *3)* 10 column volumes of methanol, sphingoids were eluted with 10 column volumes of methanol–0.4 M $CaCl_2$ 3:1 (v/v). The eluent was dried and suspended in water, and the salt was removed by a Sep-Pak C_{18} cartridge as described (21). Purified sphingoids were assayed after fluorescent tagging, using HPLC (21–25), and/or by GC as a trimethylsilyl derivative (19). The quantitative recovery of standard sphingoids examined was between 60% and 80%.

Purification of NGSL and gangliosides

The three washings of the Dowex column were pooled and dried overnight in a vacuum desiccator before acetylation as described (26). The acetylated GSL were purified through a Florisil column (0.5 × 3 cm) and washed with dichloroethane–acetone: *1)* 19:1 (v/v; discarded) and *2)* 1:1 (v/v; GSL) (26, 27). The latter fraction was deacetylated (26) and the deacetylated mixture was fractionated to NGSL and gangliosides by a DEAE-Sephadex A50 (acetate) column (0.5 × 3 cm), using chloroform–methanol–water 10:15:1 (v/v/v) and chloroform-methanol-0.06 M sodium acetate, respectively. Salt was removed from gangliosides by a Sep-Pak C_{18} cartridge (28). Each fraction was dried and stored at 4°C until further use. Other polar GSL containing sulfoglucuronic acid were eluted from the Sephadex column with 0.2 M sodium acetate. At this stage, no attempt was made to identify them [they are minor components of the CNS (29) and their concentration may be beyond the level of detection of our TLC]. Protein content was assayed with the bicinchoninic acid reagent (30).

TLC resolution of phospholipids

Phospholipid fraction 1 was resuspended in 50 μl of chloroform–methanol–water 12:7:1 (v/v/v) and 5 μl of the fraction was spotted on an HPTLC plate along with 5 μl of phospholipid fraction 2 (dissolved in chloroform-methanol-water, 4 ml/g). The plate was resolved with chloroform–ethanol–water–triethylamine 30:35:7:35 (v/v) (31) and bands were visualized with sulfuric acid-$CuSO_4$-ammonium molybdate spray (ammonium molybdate, 1 g in 93 ml of water, 2 ml of 1 M $CuSO_4$, and 5 ml of concentrated sulfuric acid) followed by heating at 110°C for 15 min. Individual bands from each lane were identified by comparison with a standard mixture. Each lane was scanned [UMAX (Fremont, CA) Power Look scanner and Adobe (San Jose, CA) Photoshop 5.1 software] and individual components were estimated as a percentage of the total.

MGC, NGSL, and gangliosides in CNS tissues

MGC, separated by a silicic acid column, were examined by TLC as follows. The sample, dissolved in chloroform–methanol 2:1 (v/v; 2–4 ml/g), was applied quantitatively (5 μl) to an HPTLC plate. The bands were resolved with chloroform–methanol–water 85:15:0.5 (v/v/v) and visualized by diphenylamine-aniline spray (32). Bands from each lane were scanned as described above and the quantity was estimated with galactosylceramide (GalCer) as the reference standard.

NGSL and gangliosides (including sulfatide) purified from the DEAE-Sephadex A50 column were examined by TLC (ganglioside fractions were spotted on a duplicate plate for quantitation of sulfatide) as described above. Chloroform–methanol–water 60:40:9 (v/v/v) and chloroform–methanol–0.25% $CaCl_2$ 55:45:10 (v/v/v) were used as solvents for NGSL and gangliosides, respectively. NGSL bands were stained by digoxigenin immunostaining (DIG-IS) (27, 33) and ganglioside bands were visualized with resorcinol spray. Both plates were scanned and each identified band (compared with a standard) was calculated as a percentage of total NGSL or ganglioside concentration. The sulfatide band was analyzed with a standard and after spraying the duplicate ganglioside-TLC plate with diphenylamine-aniline (32).

Efficacy of the recovered lipid mixture

The efficacy of the silicic column for recovery of lipid fractions was further examined by eluting a mixture of standard lipids (e.g., ceramide, ganglioside, NGSL, sulfatide, sphingoids, and SM at specific concentrations) with the solvent system described in text. The quantity of lipid fractions recovered at each step was examined and expressed as a percentage of recovered lipids.

RESULTS AND DISCUSSION

A systematic chromatographic method based on the use of a silicic acid column for purification of CNS tissue sphingolipids has been developed. Enhanced purification of individual sphingolipid (and phospholipid) components can be achieved with high recovery (**Table 1**) by eluting with a series of solvent systems that have been devised in our laboratory. Using this methodology, the sphingolipid content of bovine CNS tissue (e.g., whole brain, white matter, gray matter, and spinal cord) has been examined, using 30–50 mg of tissue. Ceramide, MGC, and GSL (and also phospholipids) were separated from the lipid extract.

TABLE 1. Recovery and assay sensitivity of sphingoid and sphingolipids by chromatographic resolution and quantification

Compounds Purified	% Recovery[a]	Assay Method	Sensitivity of Assay
Ceramide	>95	TLC/GC/HPLC	1–5 ng
Sphingoids	60–80	GC/HPLC	50–60 pg
MGC	>90	TLC	2–5 μg
NGSL	85–90	DIG-IS	2 ng
Gangliosides	85–90	DIG-IS	1 ng
Phospholipids I (cardiolipin, PE, PS, PI)	NE[b]	TLC	2–5 μg
Phospholipids II (PC and SM)	80–90	TLC	2–5 μg

[a] Examined with a standard.
[b] NE, Not examined.

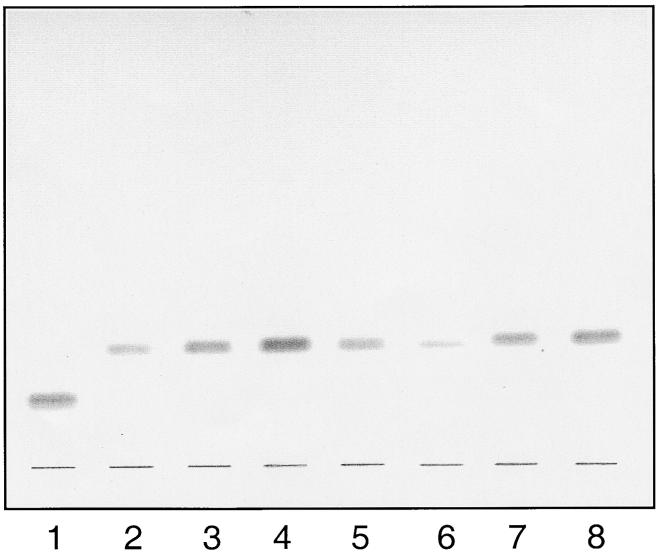

Fig. 2. Thin-layer chromatogram of the purified ceramide fractions from bovine CNS tissue. Lane 1, standard hydroxy-fatty acyl-ceramide (8 μg); lanes 2–4, standard nonhydroxy-fatty acyl-ceramide (2, 4, and 8 μg); lane 5, whole brain; lane 6, gray matter; lane 7, white matter; lane 8, spinal cord. Samples were dissoved in chloroform–methanol 2:1 (v/v; 1 ml/g tissue) and 15 μl was applied on an HPTLC plate. The bands were resolved with chloroform–methanol–acetic acid 95:4.5:0.5 (v/v/v) and visualized with benzidine spray.

NGSL and gangliosides were further purified to individual homogeneous fractions by acetylation (Florisil column) and ion-exchange (DEAE-Sephadex) chromatography. Sphingoids were purified with a Dowex column (Fig. 1) followed by Sep-Pak C_{18} cartridge chromatography (21). All purified components were assayed by a convenient single (TLC) and/or combined sensitive detection system (e.g., GC and HPLC; Table 1).

Purified ceramide from each sample was examined by TLC (**Fig. 2**) and quantitated by GC and HPLC (**Table 2**) as the trimethylsilyl and 3-keto derivative (20), respectively. Nonhydroxy-fatty acylceramide was the only free ceramide in bovine CNS tissue (Fig. 2) and no hydroxy-fatty acylceramide was observed. This is in agreement with a previous study that suggested that hydroxy fatty acyl-ceramide was glycosylated to GalCer too rapidly for detection (34). This would largely be in white matter, which is enriched in spinal cord. The higher concentration of ceramide in white matter or spinal cord than in gray matter is noteworthy.

The advantages of the method presented here over the existing diacylglycerol (DAG) kinase assay (35) (commonly used for rapid determination of ceramide, using a lipid extract and [^{32}P]ATP as substrates) are the following: the CRAQ method *1)* yields finer TLC resolution (ceramide as a single band is obtained), *2)* uses no hazardous reagent, and *3)* can easily distinguish ceramide from the biologically inactive dihydroceramide (2, 30). As an example of its value, we suggest that this is a ceramide assay alternative applicable, for example, to address the criticism of Hoffman and Dixit (36), which was directed in part at reliance on the DAG kinase assay for assessment of the postulated role of ceramide in apoptosis. Thus, the quantity of ceramide can be determined and compared with other methods after silicic acid column purification. The CRAQ method permits identification and assay of the sphingoid bases of ceramide by HPLC after removal of fatty acid by controlled acid methanolysis (14).

We have determined CNS tissue sphingoids content as fluorescent derivatives by HPLC. Existing characterization methods (21, 25) require more tissue and multiple purification steps, including serial silicic acid chromatography and TLC (25, 37) with voluminous washings and/or mild alkaline hydrolysis. The low psychosine concentration reported in murine CNS (37) may be attributed to a poor recovery of psychosine during purification and the interference of mild alkaline hydrolysis. A quantitative HPLC analysis of free sphingoid bases in developing and adult rat brain (forebrain and brainstem) and spinal cord (16–20 mg of tissue) indicated that sphingoid concentration in CNS tissue is age and region specific (38). A high sphingosine (6 ng/mg) and sphinganine (2 ng/mg) content was detected in brain and spinal cord, but no psychosine was reported in adult spinal cord (38). The purification method, prior to the HPLC analysis, included extraction, several steps of solvent partition, and washing followed by alkaline hydrolysis. We found a similar variation of sphingoid concentration in different regions of bovine brain including the enrichment of sphingoids in white matter and spinal cord (Table 2). Sphingoids were determined as orthophthalaldehyde derivatives (22–25) and trimethylsilyl derivatives (19), using HPLC and GC, respectively, to confirm their structures. A silicic acid column used at the initial step facilitated the sphingoid purification by removing lipid contaminants that interfere with Dowex chromatography. The final recovery of the sphingoids depends on the efficacy of the Dowex column because a 95% or greater recovery was achieved after the silicic acid col-

TABLE 2. MGCs, ceramide, and sphingoids in bovine CNS

Tissue	MGC	Ceramide	Psychosine	Sphingosine/Sphinganine
	mg/g tissue	*μg/g tissue*	*μg/g tissue*	*μg/g tissue*
Whole brain	7.61 ± 0.50	264 ± 79	0.28 ± 0.05	0.252 ± 0.020
Gray matter	4.10 ± 0.25	196 ± 71	0.04 ± 0.01	0.195 ± 0.030
White matter	16.65 ± 1.50	389 ± 64	1.47 ± 0.25	0.540 ± 0.040
Spinal cord	32.23 ± 2.80	421 ± 125	1.58 ± 0.30	0.710 ± 0.050

SEM of five sets of data.

umn. This has been concluded by examining individual lipid fractions as well as a mixture of standard ceramide, sphingoids, phospholipids, SM, and GSL.

MGC, the main interfering component, was removed (>90%) prior to sphingoid separation. Major lipid contaminants (e.g., GalCer, PS, and PE) were subsequently removed by a second small silicic acid column (0.5 × 6 cm). This discernibly improved HPLC resolution without the alkaline hydrolysis, which somehow interferes with the quantitative recovery of sphingoids. Mild alkaline hydrolysis affects the HPLC assay of long-chain lipid bases and their glycosylated derivative, psychosine, which has been neglected in the past. There is a demand for a sensitive and simple method to measure the free sphingoid bases without hydrolysis and from small amounts of CNS tissue (38). These requirements are met by the CRAQ procedure, which improves the method by avoiding the alkaline hydrolysis.

Psychosine recovery was dramatically increased (>90%) by eluting with a defined volume of solvent composition, chloroform–methanol–water–ammonia 120:40:2.5:3.5 (v/v/v/v), after thoroughly washing the column with 15 volumes of chloroform–methanol 9:1 (v/v). After β-galactosidase digestion, psychosine yielded two distinct peaks of sphingosine and sphinganine as resolved by HPLC. The method is superior to analysis of CNS tissue lipids, determining sphingosine in a fraction containing acidic and neutral GSL (e.g., sulfatide, ceramide monohexoside, ceramide dihexoside), neutral lipid (ceramide), and phospholipid (SM) and with sphingosine identification based on TLC-R_f alone (i.e., without chemical characterization) (9).

Ceramide and sphingoids are reported to mediate a number (and variety) of biological functions including apoptosis (2–4), while sphingosine and lysosphingolipids (e.g., psychosine) modulate transmembrane signaling (1, 4, 39, 40). Psychosine, the major sphingoid (Table 1), accumulates in the brains of patients with Krabbe's disease and is cytotoxic (21, 25). Consequently, the precise level of sphingoids in neural tissue is of great interest (41) because of their putative biological function. It is intriguing that both sphingoids and ceramide are enriched in white matter and spinal cord and that their concentration increases during critical stages of myelination (S. Dasgupta, B. Gorod, and E. L. Hogan, unpublished observations), suggesting a possible role in CNS development and myelinogenesis. We would emphasize that this improved methodology (CRAQ) enables the assay and characterization of an array of these biomolecules, which exhibit functional diversity in vitro. Further efforts to improve the sensitivity of our HPLC detection of ceramide, sphingoid, and their metabolites (e.g., sphingosine-1-phosphate) is ongoing.

MGC fractions, resolved by TLC, were scanned and the quantity of individual components was determined (**Fig. 3**). Besides GalCer, our chromatogram revealed several fast migrating bands, derivatives of GalCer, many of which are myelin components (32). They are found in gray matter at a lower concentration than in white matter or spinal cord (Fig. 3). These major brain MGC components are

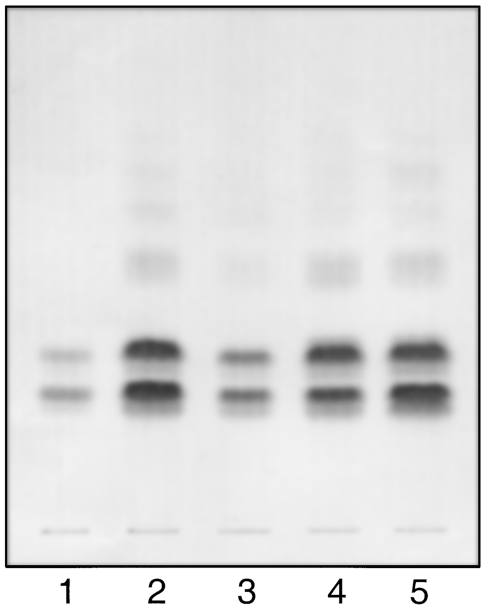

Fig. 3. Thin-layer chromatogram of the purified MGC fractions from bovine CNS tissue. Lane 1, standard GalCer; lane 2, whole brain; lane 3, gray matter; lane 4, white matter; lane 5, spinal cord. Samples were dissolved in chloroform–methanol (v/v; 2–4 ml/g tissue), spotted (5 μl), and developed in chloroform–methanol–water 85:15:0.5 (v/v/v). The bands were visualized with diphenylamine-aniline spray and scanned.

presumably acyl and ether (or acetal) derivatives of glycosyl hydroxyl groups of GalCer and GlcCer, and are found in adults of several species (41–45). They are mainly alkali labile (although some are acid susceptible) and are hydrolyzed to GalCer (32). They will be lost if GSL are purified after alkaline methanolysis followed by acid neutralization, and hence are a tissue molecular species that has been neglected previously (9). It is anticipated that nonpolar plasmalocerebrosides and plasmalopsychosines, previously characterized in human brain (41–43), may be eluted in the MGC fraction from the silicic acid column. The MGC fraction is now being carefully examined and its detailed characterization will be reported elsewhere. It is noteworthy that this study using purified myelin from bovine spinal cord indicated a 3-fold, 1.5-fold, 2-fold, and 3-fold tentative increase in nonpolar GalCer derivatives (MGC), ceramide, sphingosine, and psychosine content, respectively, in myelin compared with spinal cord and, thus, further supports our hypothesis that these compounds may have a unique role in myelin structure and function.

NGSL, freed from contaminating lipids (e.g., nonpolar lipids, phospholipids and gangliosides) by Florisil and DEAE-Sephadex chromatography, were examined by DIG-IS (**Fig. 4**) (27, 33). In addition to the previously defined brain NGSL, a tetraglycosylceramide, $GgOse_4Cer$ (20, 46); three pentaglycosylceramides, $GbOse_5Cer$ (47), Lewis X, and other long-chain NGSL of the Lewis X-series (27); and a novel GalNAc-GA1 (45) have been characterized. The CRAQ method will enable the characterization of several other, probably novel NGSL in CNS tissue that are revealed by DIG-IS. In contrast to the report by Dreyfus et al.

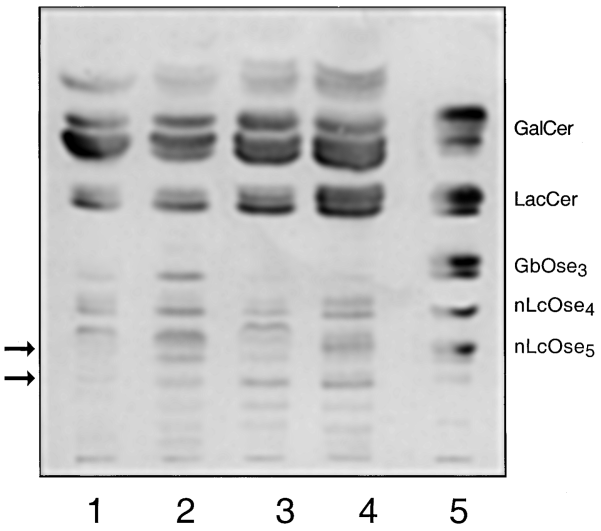

Fig. 4. NGSL profile of bovine CNS tissue. Lane 1, whole brain; lane 2, gray matter; lane 3, white matter; lane 4, spinal cord; lane 5, standard NGSL. Samples were dissolved in chloroform–methanol–water 12:7:1 (v/v/v; 0.5 ml/g tissue), and 15 μl of the sample was spotted and resolved, using chloroform–methanol–water 60:40:9 (v/v/v) and visualized by DIG-IS as described in text.

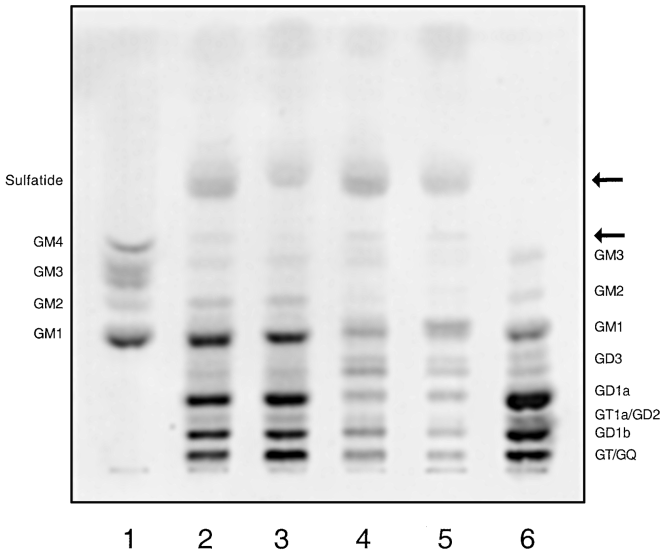

Fig. 5. Thin-layer chromatogram of bovine CNS gangliosides. Lane 1, standard brain monosialoganglioside; lane 2, whole brain lane; lane 3, gray matter; lane 4, white matter; lane 5, spinal cord; lane 6, standard brain mono- and disialoganglioside. Samples were dissolved in choloform–methanol–water 12:7:1 (v/v/v; 1 ml/g tissue), 15 μl was spotted on an HPTLC plate, and resolved with chloroform–methanol–0.25% $CaCl_2$ 55:45:10 (v/v/v). The bands were visualized with resorcinol-HCl spray.

(9), the chromatogram is free of contaminating lipid, for example, ceramide and phospholipid, and the resolution now achieved allows for fine distinction. For example, in comparing the NGSL composition of white matter and spinal cord with gray matter (Fig. 4, marked with arrows), there is a specificity to tissue and cell type of long-chain oligoglycosyl minor NGSL. The bands in white and gray matter, respectively, have been characterized by TLC-immunostaining as GA1 (myelin specific) and GalNAc-GA1 (neuron specific), respectively, using monospecific antibody (11, 46, 48).

The ganglioside and sulfatide content in each tissue fraction can be quantitated with appropriate standards (results not shown). These data confirm that sulfatide and GM4 are enriched in white matter and spinal cord (**Fig. 5**, lanes 4 and 5; marked with arrows) and are myelin components. The other gangliosides identified are GD1a, GD1b, and GT1b, which, along with GM1, are prominent in gray matter. The ganglioside profile of whole brain (Fig. 5, lane 2) is the sum of findings in white matter and gray matter and is consistent with previous studies of human, cat, and rabbit gangliosides of white matter, gray matter, and spinal cord (49–51). According to Ledeen (50), the ganglioside distribution in human CNS tissue (determined by sialic acid content per gram of wet tissue) is as follows: gray matter (900 μg) >> white matter (267 μg) >> spinal cord (87 μg). A similar profile was found in our study of bovine tissue. Using this CRAQ methodology, we have identified and quantitated gangliosides of CNS that correspond well with previous studies of vertebrate CNS gangliosides. The sensitivity of the ganglioside identification can be amplified (1–10 pmol) by using DIG-IS (33).

PC and SM, the major CNS phospholipids along with cardiolipin and PE (**Fig. 6**), were eluted as a single fraction (methanol elution) while the phospholipids cardiolipin, PE, PS, and PI were eluted along with the GSL (THF-water). Our attempt to recover these phospholipids from the Florisil column, as proposed previously (26), was unsuccessful (results not shown), indicating that degrada-

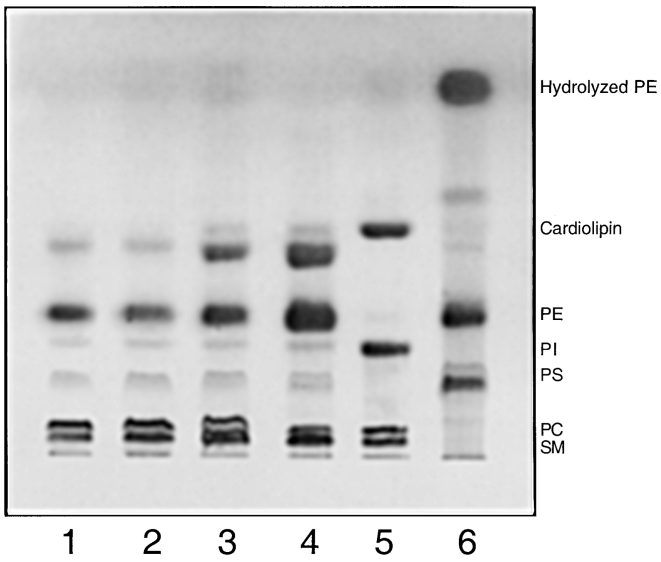

Fig. 6. Thin-layer chromatogram of bovine CNS phospholipid. Lane 1, whole brain; lane 2, gray matter; lane 3, white matter; lane 4, spinal cord; lane 5, standard PI, PC, and SM; lane 6, standard PE and PS. Five μl of phospholipid fraction 1 (15 ml/g tissue) and phospholipid fraction 2 (4 ml/g tissue) from each sample were spotted on an HPTLC plate, the bands were resolved in chloroform–ethanol–water–triethylamine 35:30:7:35 (v/v/v/v), and visualized with $CuSO_4$-H_2SO_4-molybdate spray.

tion occurs during acetylation. Phospholipid bands were scanned and estimated as a percentage of total concentration: cardiolipin and PE were the major myelin phospholipids. None of the purified lipid fractions contained contaminating lipid except for the primary THF-water elution, which contained both GSL (purified by Florisil column after acetylation) and phospholipids that were resolved with a suitable solvent composition (31). An excellent purification and recovery of SM (and also PC), a major source of in vivo ceramide (2), was achieved. Because this method has been developed primarily for the purification and quantitative resolution of tissue sphingoids and sphingolipids, phospholipids other than SM and PC, which are not directly metabolically related to sphingolipids, were not explored in detail. SM can also be estimated by HPLC after fluorescent labeling (20). The chloroform wash of the silicic acid column contains the nonpolar lipids (free fatty acids, glycerolipids, cholesterol etc.), which may be further resolved analytically (9).

Another method (51) for determination of sphingolipid metabolites using simple extraction and modified Folch's partition (52) describes quantitation of ceramide, sphingoids, sphingomyelin, and sphingophosphorylcholine in HL-60 cells by tandem mass spectrometry. It has the following limitations: *1)* GSL and phospholipids other than SM cannot be examined, and *2)* the method was not applied to biological tissue in vivo. High concentrations of other lipids in in vivo tissue renders analysis more complicated than that of cultured cell extracts.

In conclusion, the CRAQ methodology for purification of CNS tissue sphingoids and sphingolipids utilizes silicic acid and Dowex chromatography under neutral conditions, and introduces new solvent compositions. The purified individual sphingolipids, including sphingoids, are resolved quantitatively by TLC, GC, and HPLC. Sphingoids, although minor CNS components, are believed to play critical roles in regulation of cell development and apoptosis. It is worth mentioning that our examination of the sphingolipid profile of nonneuronal tissues (kidney, liver, heart, spleen, and erythrocytes) and cultured oligodendrocytes (prepared by N. Bhat, Department of Neurology, Medical University of South Carolina, Charleston, SC) indicates a similar quantitative resolution and recovery (S. Dasgupta, E. L. Hogan, and N. Bhat, unpublished observations). The CRAQ methodology provides a valuable tool for quantitation of sphingoids and sphingolipids in tissue and cells and is useful for the study of sphingolipid metabolites in relation to their precise biological structure and functions.

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REFERENCES

1. Hannun, Y. A., and R. M. Bell. 1989. Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science.* **243:** 500–507.
2. Hannun, Y. A. 1994. The sphingomyelin cycle and second messenger function of ceramide. *J. Biol. Chem.* **269:** 3125–3128.
3. Mathias, S., L. A. Pena, and R. Kolesnick. 1998. Signal transduction of stress via ceramide [review article]. *Biochem. J.* **335:** 465–480.
4. Hannun, Y. A., and R. M. Bell. 1987. Lysosphingolipids inhibit protein kinase C: implication for sphingolipidosis. *Science.* **235:** 670–674.
5. Hakomori, S-i. 1990. Bifunctional role of glycosphingolipids: modulators for transmembrane signaling and mediators for cellular interactions. *J. Biol. Chem.* **265:** 18713–18716.
6. Yavin, E., and W. H. Habig. 1984. Binding of tetanus toxin to somatic neutral hybrid cells with varying ganglioside composition. *J. Neurochem.* **42:** 1313–1320.
7. Yu, R. K., L. J. Macala, T. Taki, H. M. Winfield, and F. S. Yu. 1988. Developmental changes in ganglioside composition and synthesis in embryonic rat brain. *J. Neurochem.* **50:** 1825–1829.
8. Daniotti, J. L., C. A. Landa, D. Gravotta, and H. J. Maccioni. 1990. GD3 ganglioside is prevalent in fully differentiated neurons from rat retina. *J. Neurosci. Res.* **26:** 436–446.
9. Dreyfus, H., B. Guerold, L. Freysz, and D. Hicks. 1997. Successive isolation and separation of the major lipid fractions including gangliosides from single biological samples. *Anal. Biochem.* **249:** 67–78.
10. Dasgupta, S., J. Glushka, H. van Halbeek, and E. L. Hogan. 1994. Branched monosialo gangliosides of the lacto-series isolated from bovine erythrocytes: characterization of a novel ganglioside, NeuGc-isoOctaosylceramide. *Arch. Biochem. Biophys.* **310:** 373–384.
11. Dasgupta, S., H. van Halbeek, and E. L. Hogan. 1992. Ganglio-*N*-tetraosyl ceramide (GA1) of bovine and human brain: molecular characterization and presence in myelin. *FEBS Lett.* **301:** 141–144.
12. Motta, S., M. Mouti, S. Sesana, R. Caputto, C. S. Arelli, and R. Ghidoni. 1993. Ceramide composition of the psoriatic scale. *Biochim. Biophys. Acta.* **1182:** 147–151.
13. Bischel, M. D., and J. H. Austin. 1963. A modified benzidine method for the chromatographic detection of sphingolipids and acid polysaccharides. *Biochim. Biophys. Acta.* **70:** 598–600.
14. Sweeley, C. C., and B. Siddiqui. 1977. Chemistry of mammalian glycolipids. *In* The Glycoconjugates. M. I. Horowitz and W. Pigman, editors. Academic Press, New York. 459–540.
15. Vance, D. E., and C. C. Sweeley. 1967. Quantitative determination of the neutral glycosylceramides in human blood. *J. Lipid Res.* **8:** 621–630.
16. Yu, R. K., and R. W. Ledeen. 1970. Gas-liquid chromatographic assay of lipid-bound sialic acids: measurement of gangliosides in brain of several species. *J. Lipid Res.* **11:** 506–516.
17. Rauvala, H., and J. Karkkainen. 1977. Methylation analysis of neuraminic acids by gas chromatography-mass spectrometry. *Carbohydr. Res.* **56:** 1–10.
18. Kawamura, N., and T. Taketomi. 1977. A new procedure for the isolation of brain gangliosides, and determination of their long chain base compositions. *J. Biochem. Tokyo.* **81:** 1217–1225.
19. Gaver, R. C., and C. C. Sweeley. 1965. Methods for methanolysis of sphingolipids and direct determination of long-chain bases by gas chromatography. *J. Oil Chem. Soc.* **42:** 294–298.
20. Iwamori, M., H. W. Moser, R. H. McCluer, and Y. Kishimoto. 1975. 3-Ketosphingolipids: application to determination of sphingolipids which contain 4-sphingenine. *Biochim. Biophys. Acta.* **380:** 308–319.
21. Igishu, H., and K. Suzuki. 1984. Analysis of galactosphingosine (psychosine) in the brain. *J. Lipid Res.* **25:** 1000–1006.
22. Naoi, M., Y. C. Lee, and S. Roseman. 1974. Rapid and sensitive determination of sphingosine base and sphingolipids with fluorescamine. *Anal. Biochem.* **58:** 571–577.
23. Kisic, A., and M. M. Rapport. 1974. Determination of long-chain base in glycosphingolipids with fluorescamine. *J. Lipid Res.* **15:** 179–180.
24. Higgins, T. J. 1984. Simplified fluorometric assay for sphingosine bases. *J. Lipid Res.* **25:** 1007–1009.
25. Shinoda, H., T. Kobayashi, M. Katayama, I. Goto, and H. Nagara. 1987. Accumulation of galactosylsphingosine (psychosine) in the twitcher mouse: determination by HPLC. *J. Neurochem.* **420:** 411–416.
26. Saito, T., and S-i. Hakomori. 1971. Quantitative isolation of total glycosphingolipids from animal cells. *J. Lipid Res.* **12:** 257–259.
27. Dasgupta, S., E. L. Hogan, and S. S. Spicer. 1996. Stage-specific expression of fuconeolacto- (Lewis X) and ganglio-series neutral glycosphingolipids during brain development: characterization of

Lewis X and related glycosphingolipids in bovine, human and rat brain. *Glycoconj. J.* **13:** 367–375.
28. Williams, M. A., and R. H. McCluer. 1980. The use of Sep-Pak cartridge during the isolation of ganglioside. *J. Neurochem.* **35:** 266–269.
29. Chou, D. K., and F. B. Jungalwala. 1993. *N*-Acetylglucosaminyltransferase regulates the expression of neolactoglycolipids including sulfoglucuronylglycolipids in the developing nervous system. *J. Biol. Chem.* **268:** 21727–21733.
30. Shibuya, T., Y. Watanabe, K. A. Nalley, A. Fusco, and B. Salafsky. 1989. The BCA protein determination system: an analysis of several buffers, incubation temperature and protein standards. *Tokyo Ika Daigaku Zasshi* **47:** 571–577.
31. Leray, C., and X. Pelletier. 1987. Thin-layer chromatography of human platelet phospholipids with fatty acid analysis. *J. Chromatogr.* **420:** 411–416.
32. Dasgupta, S., M. B. Everhart, N. Bhat, and E. L. Hogan. 1997. Neutral monoglycosylceramides in rat brain: occurrence, molecular expression and developmental variation. *Dev. Neurosci.* **19:** 152–161.
33. Kniep, B., and P. F. Muhlradt. 1990. Immunochemical detection of glycosphingolipids on thin-layer chromatograms. *Anal. Biochem.* **188:** 5–8.
34. Radin, N. S. 1994. Glucosylceramide in the nervous system—a mini-review. *Neurochem. Res.* **19:** 533–541.
35. Welsh, N. 1996. Interleukin-1β-induced ceramide and diacylglycerol generation may lead to activation of c-Jun NH_2 terminal kinase and the transcription factor ATF2 in the insulin-producing cell line Rlm5F. *J. Biol. Chem.* **271:** 8307–8312.
36. Hoffman, K., and V. M. Dixit. 1998. Ceramide in apoptosis—does it really matter? *Trends Biochem. Sci.* **23:** 374–377.
37. Kobayashi, T., K. Mitsu, and I. Goto. 1988. Free sphingoid bases in normal murine tissues. *Eur. J. Biochem.* **172:** 747–752.
38. Kwon, O-S., G. D. Newport, and W. Slikker. 1998. Quantitative analysis of free sphingoid bases in brain and spinal cord tissues by high-performance liquid chromatography with a fluorescent detection. *J. Chromatogr.* **720:** 9–14.
39. Merrill, A. H., and V. L. Stevens. 1989. Modulation of protein kinase C and diverse cell functions by sphingosine: a pharmacologically interesting compound linking sphingolipids and signal tranduction. *Biochim. Biophys. Acta.* **1010:** 131–139.
40. Zhang, Y., and R. Kolesnick. 1995. Signaling through the sphingomyelin pathway. *Endocrinology.* **136:** 4157–4160.
41. Nudelman, E. D., S. B. Levery, Y. Igarashi, and S-i. Hakomori. 1992. Plasmalopsychosine, a novel plasmal (fatty aldehyde) conjugate of psychosine with cyclic acetal linkage: isolation and characterization from human brain white matter. *J. Biol. Chem.* **267:** 11007–110016.
42. Levery, S. B., E. D. Nudelman, and S-i. Hakomori. 1992. Novel modification of glycosphingolipids by long-chain cyclic acetals: isolation and characterization of plasmalocerebroside from human brain. *Biochemistry.* **31:** 5335–5340.
43. Sadozai, K. K., S. B. Levery, J. K. Anand, and S-i. Hakomori. 1996. Model compounds for plasmalo glycolipids: preparation of long chain cyclic acetals of methyl β-D-galactopyranoside and determination of their regio- and stereochemistry by proton NMR. *J. Carbohydr. Chem.* **15:** 715–725.
44. Kishimoto, Y., M. Wazda, and N. S. Radin. 1968. 6-Acyl-galactosylceramides of pig brain: structure and fatty acid composition. *J. Lipid Res.* **9:** 27–33.
45. Dasgupta, S., and E. L. Hogan. 1993. Molecular characterization of gangliotetraosylceramide (GA1) in normal human brain and its developmental changes. *Ind. J. Biochem. Biophys.* **30:** 341–345.
46. Dasgupta, S., E. L. Hogan, and H. van Halbeek. 1995. Chemical and immunological characterization of galactosyl β1-3-globoside in bovine, human and rat brain. *J. Neurochem.* **65:** 2344–2349.
47. Dasgupta, S., H. van Halbeek, S. S. Spicer, and E. L. Hogan. 2000. Molecular characterization and immunochemical localization of $IV^4GalNAcGgOse_4Cer$; a naturally occuring novel neutral glycosphingolipid in bovine brain. *Glycobiology.* **10:** 1–9.
48. Ueno, K., S. Ando, and R. K. Yu. 1978. Gangliosides of human, cat, and rabbit spinal cord and cord myelin. *J. Lipid Res.* **19:** 863–871.
49. Ledeen, R. W., and R. K. Yu. 1982. Gangliosides: structure, isolation and analysis. *Methods Enzymol.* **83:** 139–191.
50. Ledeen, R. W. 1979. Structure and distribution of gangliosides. *In* Complex Carbohydrates of Nervous Tissues. R. W. Margolis and R. K. Margolis, editors. Plenum Press, New York. 1–23.
51. Mano, N., Y. Oda, K. Yamada, N. Asakawa, and K. Katayama. 1997. Simultaneous quantitative determination method for sphingolipid metabolites by liquid chromatography/ionspray ionization tandem mass spectrometry. *Anal. Biochem.* **244:** 291–300.
52. Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.