Rapid, sensitive spectrophotometric method for quantitative determination of sulfatides

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ABSTRACT A rapid, sensitive spectrophotometric method for the analysis of sulfatides is described. The assay method is based upon the formation of a colored complex between the cationic dye azure A and (anionic) sulfolipids; the complex is extractable by a solution of chloroform–methanol 1:1. With the exception of some of the phospholipids, the reaction is specific for the sulfolipids. Cardiolipin was the most reactive of the non-sulfolipid compounds that were tested. Except for sphingosine and the mono- and dihexose derivatives of sphingosine, a wide variety of lipids did not interfere with the formation of color by standard sulfatides. Sulfur-containing amino acids, sugar sulfates, and sulfated polymers neither produced color nor interfered in its formation by sulfatide. Chloride and nitrate ions produced relatively little color.

The method is much more sensitive (lower limit, about 0.002 μmole) than most methods currently employed for the analysis of sulfatides and has a high degree of precision. It is applicable to the analysis of sulfolipids in tissue extracts and gives values similar to those obtained by previously published procedures. The reliability of the method is increased when it is applied to partially purified lipid solutions. Sulfatides added to extracts of rabbit brain tissue were quantitatively accounted for by the assay. In addition, the method can be applied to samples obtained directly from thin-layer plates.

KEY WORDS colorimetric analysis · azure A · cerebroside sulfate · brain · sulfolipid · sulfatide · cholesteryl sulfate · fatty alcohol sulfates · gray matter · white matter · rabbit

MANY STUDIES HAVE BEEN CONDUCTED in recent years dealing with the occurrence, distribution, and metabolism of the sulfatides (cerebroside sulfates) in mammalian tissues (1, 2), and several methods for their quantitative determination have been published. Most procedures are based on the measurement of sulfate liberated by acid hydrolysis: Davison and Gregson (3) utilized the turbidimetric method of Dodgson (4) to estimate the liberated sulfate; Green and Robinson (5) adapted the barium chloranilate procedure of Bertolacini and Barney (6); Long and Staples (7) measured the excess 4-amino-4′-chlorodiphenyl that remained after reaction with the liberated sulfate ion in the method of Jones and Letham (8). Sulfate content was determined by Mårtensson (9) after reduction to sulfide, which was then measured as a bismuth sulfide sol. After mineralization of sulfatides by Carius digestion, Stoffyn and Keane (10) also utilized the barium chloranilate procedure.

There are several limitations inherent in these methods. When applied to the analysis of the sulfatide content of tissue extracts the determinations are subject to error if nonlipid sulfates are present. Most of the methods require either lengthy or carefully controlled hydrolytic techniques. The use of specialized glassware and the necessity for removing interfering cations are added limitations for the routine utilization of some of these procedures. In addition, most of the methods suffer from a lack of sensitivity that precludes their use for the determination of the sulfatide content in small amounts of tissue or in tissues having relatively low concentrations of lipid sulfates. The method of Stoffyn and Keane can be used to measure about 0.01 μmole of sulfatide; the lower limit of the other methods is about 0.1 μmole.

In 1945, Jones (11) observed that sulfonated surface-active compounds form, with methylene blue, colored salts that are extractable into chloroform. This observation was the basis of a simple method for the quantitative determination of small amounts of anionic surfactants. Vlitos (12) utilized a similar reaction for the determination of dichlorophenoxyethyl sulfate in soil and this procedure was adapted by Roy (13) and modified by Crépy

Abbreviations: C–M, chloroform–methanol (v/v); TLC, thin-layer chromatography; FA, fatty acids.

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and Rulleau-Meslin (14) for the estimation of steroid sulfates. Haines (15) refers to the use of this reaction in the study of microbial sulfolipids. The specificity of this type of reaction for the lipid sulfates in the presence of other materials usually present in lipid extracts of tissues has not been reported, nor has the accuracy of these procedures been compared with that of other methods for the estimation of lipid sulfates. In addition, this type of reaction has not as yet been applied to the analysis of sulfatides.

Recently, van Steveninck and Riemersma (16) observed that another thionine dye, azure A, had several advantages over methylene blue when applied to the analysis of surfactants, primarily its relative insensitivity to interference from various cations.

The present work describes a rapid nonhydrolytic method for the quantitative estimation of lipid sulfates that is based upon the ability of these materials to form colored azure A complexes that are extractable into chloroform–methanol solutions. Sulfatide was used as the primary lipid sulfate standard in these studies. The specificity, sensitivity, and reproducibility of this method are described. Its accuracy as compared to other methods for the determination of lipid sulfates in extracts of brain tissue has been investigated as well as its ability to measure sulfatide added to a variety of purified lipids and to extracts of brain white and gray matter. The procedure cannot distinguish between classes of lipid sulfates such as the steroid sulfates or the glycolipid sulfates when these are present as mixtures, but this selectivity can be achieved when these compounds are assayed directly after separation by thin-layer chromatography.

MATERIALS

The following compounds were obtained from commercial sources (the fatty acid residue that was used as the basis for calculating molecular weights is given in parentheses). Mann Research Labs Inc., New York: sphingomyelin (palmitoyl), "DL-α-cephalin dipalmitoyl" (a synthetic phosphatidyl ethanolamine), DL-α-lecithin dipalmitoyl (a synthetic phosphatidyl choline), phosphatidyl serine (distearoyl), cholesterol, tripalmitin, sodium hexyl sulfate, sodium octyl sulfate, sodium decyl sulfate, sodium dodecyl sulfate, sodium tetradecyl sulfate, sodium tetradecyl sulfonate, L-cystine, and hyaluronic acid. Pierce Chemical Co., Rockford, Ill.: phosphatidyl ethanolamine (dipalmitoyl) and sphingosine sulfate. Applied Science Laboratories Inc., State College, Pa.: "ceramide[1] (nonhydroxy FA)" (lignocerate) and "ceramide (hydroxy FA)" (cerebronate). Miles-Yeda Research and Development Co., Ltd., Rehovoth, Israel: dihydrosphingosine, cerasine (*N*-lignoceroyl-DL-sphingosyl-β-D-galactoside), cytolipin H (lignocerate), and lactosyl sphingosine. Sigma Chemical Co., St. Louis, Mo.; estrone-3-sulfate (potassium salt), chondroitin sulfate (grade III). Ikapharm, Ramat-Gan, Israel: estriol trisulfate (trisodium salt). General Biochemicals, Chagrin Falls, Ohio: cholesteryl sulfate (sodium salt), L-cysteine, phosphatidic acid (dipalmitoyl, disodium), and cardiolipin. Calbiochem, Los Angeles, Calif.: L-cysteic acid, *S*-ethyl-L-cysteine and protamine sulfate. Nutritional Biochemical Corporation, Cleveland, Ohio: DL-methionine and methionine sulfoxide. Eastman Kodak Co., Rochester, N.Y.: adenine sulfate. Medical Research Laboratories, University of Toronto, Toronto, Canada: heparin (sodium salt), 150.9 USP units/mg. Supelco, Inc., Bellefonte, Pa.: bovine sulfatides (the major fatty acid in this preparation was stearic acid and the molecular weight was calculated on this basis). National Aniline Division of Allied Chemical Corp., New York: azure A (total dye content, 92%),[2] azure B (total dye content, 84%), azure C (total dye content, 75%), and methylene blue (total dye content, 87%). Fisher Scientific Company, Fairlawn, N.J.: azure A (total dye content, 89%). Aldrich Chemical Co., Inc., Milwaukee, Wis.: azure A. Matheson, Coleman and Bell, Cincinatti, Ohio: azure A (total dye content, 77%). Harleco, Philadelphia, Pa.: azure C.

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Sphingosine and ceramide were also prepared by the method of Tipton (18). Florisil was obtained from the Floridin Co., Tallahassee, Fla. All solvents and other chemicals used in this study were reagent grade. Screw-

[1] The nomenclature used in this report is as follows: ceramide, *N*-acyl sphingosine; psychosine, galactosido-sphingosine; cytolipin H, ceramide lactoside.

[2] This was the dye preparation used for most of the studies reported here.

[3] This material is a sulfated form of hyaluronic acid, not naturally occurring, which produces vitreous liquefaction and retinal detachment in rabbits (30).

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cap culture tubes (16 × 125 mm, supplied with polypropylene caps) used for the colorimetric analyses were obtained from Demuth Glass Division, Brockway Glass Co., Inc., Parkersburg, W. Va.

METHODS

Analytical Procedures

The Azure A Colorimetric Assay. Samples containing 0.0020–0.035 μmole of sulfatide were pipetted into screw-cap culture tubes and evaporated to dryness. To each tube was added 5.0 ml of C–M 1:1, 5.0 ml of 0.05 N H_2SO_4, and 1.0 ml of dye solution. (The dye solution was prepared as follows: 40 mg of azure A plus 5.0 ml of 0.05 N sulfuric acid were diluted to 100 ml with water; the dye solution was stored in a dark bottle, at room temperature, and was stable for at least 1 wk.) The tubes were capped, shaken for 30 sec, and centrifuged at room temperature for 5 min at about 300 *g*. The absorbance of the lower phase was measured on a Beckman spectrophotometer at 645 mμ, in microabsorption cells of 0.9 ml capacity and 10 mm light path.

Sulfate, Galactose, Nitrogen. The sulfate content of standard sulfatides and that of tissue extracts were determined. Samples were hydrolyzed according to the procedure of Green and Robinson (5) (sealed tube) or according to the method of Davison and Gregson (3) (reflux). The hydrolysis mixtures were evaporated to dryness in a vacuum desiccator over KOH, the residue was dissolved in water, and the liberated sulfate ion was then extracted by the procedure of Folch, Lees, and Sloane Stanley (19). The upper phase was evaporated to dryness and its sulfate content determined by turbidimetric means according to procedure B of Dodgson (4) as modified by Davison and Gregson (3). Standard solutions of K_2SO_4 and the sulfatide preparation received from Dr. Stoffyn were carried throughout the procedures.

Galactose was determined according to the procedure of Radin, Lavin, and Brown (20).

Total nitrogen was determined by the micro-Kjeldahl procedure of Kabat and Mayer (21).

Thin-Layer Chromatography

Silica Gel G (Brinkmann Instruments Inc., Westbury, N.Y.) was applied to plates with a Desaga/Brinkmann adjustable applicator as a slurry 250 μ thick (30 g of silica gel per 65 ml of water). The plates were activated by heating for 1 hr at 125°C and stored in a desiccator until use. The following solvent systems were used (all v/v): A, chloroform–methanol–water 65:25:4 (22); B, *n*-propanol–concentrated NH_4OH–water 12:1:2 (23); C, chloroform–methanol–concentrated NH_4OH 80:20:0.4 (24). Materials on the chromatograms were detected by charring according to the procedure of Privett and Blank (25). When materials were to be recovered from thin-layer plates, they were detected by exposure of the dry plates to iodine vapor for 1 min.

Lipid Extraction

Adult albino rabbits were killed by air embolism. The brains were immediately removed and dissected in the cold. Lipids were extracted by homogenization with C–M 2:1 (20 ml/g of tissue) in a conical glass homogenizer (Kontes Glass Co., Vineland, N.J.). The C–M extract (crude extract) was filtered through solvent-washed filter paper and the filtrate was washed according to the method of Folch et al. (19), with 0.1 M KCl as the aqueous phase. The lower phase, washed with "pure solvents upper phase" prepared with 0.1 M KCl, was evaporated to dryness in tarred vessels, stored in a desiccator over KOH under vacuum, and weighed ("Folch-washed extract").

Florisil Chromatography

Florisil was prepared according to the method of Wells and Dittmer (26). Florisil chromatography was employed to separate the sulfatides from cholesterol, ceramide, and the phospholipids. The lipid extract after the Folch washing was transferred to columns of Florisil and fractionated according to the method of Rouser, Bauman, Kritchevsky, Heller, and O'Brien (27). Cerebrosides and sulfatides were eluted in the C–M 2:1 fraction, as shown by TLC.

Radioactivity Studies

Rabbits were injected intravenously with $Na_2{}^{35}SO_4$ (0.4 μc/g of body weight). After 1 wk the animals were killed. The tissues were extracted and the lipids fractionated as indicated previously. The Folch washing procedure was carried out until no counts remained in the last upper phase wash. Radioactivity was measured by liquid scintillation counting in a Packard 314X Tri-Carb scintillation spectrometer. The lipid extracts were dried in counting vials and counted in a toluene system containing 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter of toluene; aqueous solutions were counted in a dioxane system (28). The counting efficiency was about 59%. All samples were corrected for quenching by internal standardization and counts were corrected for decay back to the time of injection of isotope into the animal.

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RESULTS

Standard Sulfatides

The sulfatide obtained from Supelco gave the following analytical values (in μmoles/ml, mean ± SEM for the number of determinations shown): sulfate, after sealed tube hydrolysis, 1.05 ± 0.01 (n = 3), and after hydrolysis under reflux conditions, 0.958 ± 0.013 (n = 2); galactose, 0.90 ± 0.03 (n = 3); total nitrogen, 0.95 ± 0.05 (n = 2). These components are present, then, in essentially equimolar proportions, which is the expected relationship. The compound had a galactose content of 20.3% (theoretical, 22.3%); sulfate, 12.5% (theoretical, 11.9%); and nitrogen, 1.65% (theoretical, 1.73%). The sulfatide received from Dr. Stoffyn gave the following values: sulfate (sealed tube hydrolysis), 0.521 μmole/ml; galactose, 0.548 ± 0.012 (n = 3) μmole/ml —again, essentially equimolar ratios of these moieties.

TLC showed that these two compounds had identical migration patterns with the three solvent systems described above. These studies verified the purity of the Supelco product, which was used as the standard for further studies.

Characteristics of the Azure A Colorimetric Assay

Spectrum, Beer's Law Adherence, and Precision. Examination of the spectrum between 590 and 690 mμ of the azure A complex formed by sulfatide revealed a peak of 647 mμ. Routine analyses were performed at 645 mμ.

The absorbance of the color was proportional to the amount of sulfatide used in the assay over the range from 0.002 to 0.035 μmole (Fig. 1).

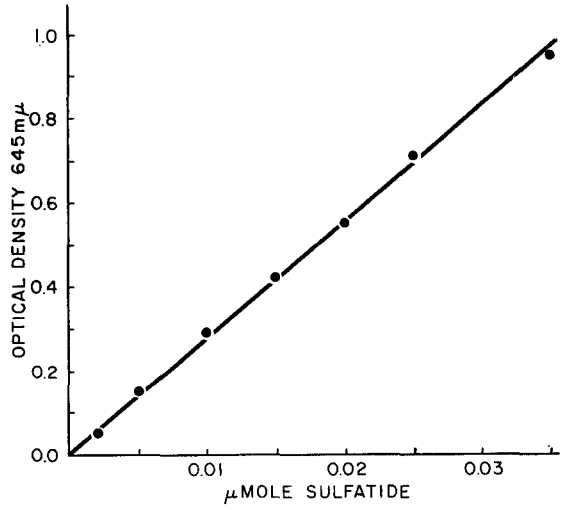

FIG. 1. Proportionality between the absorbance of the colored complex formed and the amount of sulfatide used in the azure A colorimetric assay. The optical density was measured at 645 mμ on a Beckman DU spectrophotometer.

The optical density in 10 analyses of the same solution of sulfatide was 0.541 ± 0.002, which indicates high reproducibility for the method. Eighteen standard curves prepared over a period of several weeks by different operators showed about 1% variation in the molar extinction coefficient: (6.88 ± 0.072) × 10^4.

Color Stability and Shaking Time. The color was stable for about 4 hr, when measured in glass-stoppered cuvettes, with about 10% decrease in optical density over the next 24 hr. No differences were observed in samples protected from the light. Occasionally, the organic phase became cloudy, but cleared within 2 min after being transferred to cuvettes. No differences were observed in the amount of color extracted when the tubes were shaken for periods from 30 sec to 5 min.

Effect of Methanol Concentration. The absorbance of the azure A–sulfolipid complex that was extracted into the organic phase was dependent upon the ratio of chloroform to methanol used in the extraction procedure. There was about a six-fold increase in absorbance of the lower phase after extraction with C–M 1:1 as compared to the chloroform extract. This is a function both of a concentration effect (about a two-fold decrease in volume of the organic phase from chloroform to C–M 1:1) and an enhancement of color due to the presence of the alcohol. This increase in optical density per ml of organic phase is depicted in Fig. 2. The ratio of chloroform to methanol of 1:1 selected for the routine analyses gives a volume convenient for measurement (2.50 ml). Higher absorbancies were obtained with increasing concentrations of meth-

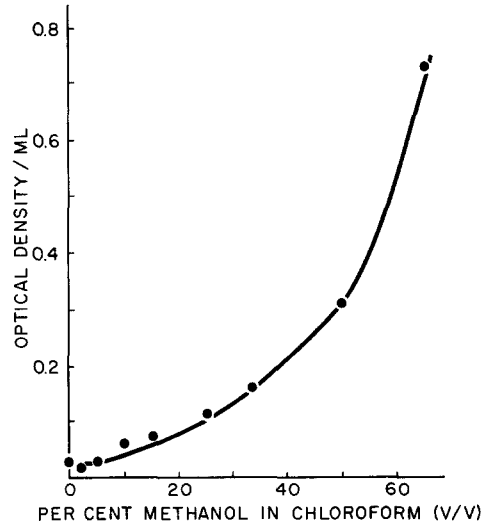

FIG. 2. Effect of the percentage of methanol in the chloroform–methanol used for extracting the colored complex formed between azure A and sulfatide (NB: this does not indicate the composition of the lower layer obtained after mixing). The ordinate represents OD/ml of organic phase after the reaction of 0.025 μmole of sulfatide as described under Methods. The absorbancy was measured at 645 mμ against blanks prepared with the corresponding chloroform–methanol mixtures.

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anol, although the blank becomes more blue after the C–M 1:1 stage. The same spectrum was obtained for all of the solutions analyzed when measured against blanks prepared with the corresponding C–M solutions.

Effects of Various Dyes, Aqueous Phases, and Ions. There was less than 5% variation in absorbance of the color formed by sulfatide when concentrations of azure A from 0.02 to 0.05% were used. A dye concentration of 0.04% was selected for routine use. Several other thionine dyes, prepared in the same manner as azure A in the standard procedure, were tested for the colorimetric analysis of sulfatides. Azure B, azure C, and methylene blue, all from Allied Chemicals, gave the same color yield as azure A. Azure C (from Harleco) gave about 15% lower absorbancy. Azure A samples supplied by both Matheson, Coleman, and Bell and Aldrich gave the same color yield as did that from Allied Chemicals, while that from Fisher gave about half this value. The reason for the lower values obtained with this latter preparation is not known, but exemplifies a variability in dye preparations against which precautions should be taken.

The choice of 0.05 N sulfuric acid as the aqueous phase was somewhat arbitrary since there was less than 10% variation in color yield when the sulfuric acid concentration was varied from 0.01 N to 1.0 N. Water, in the place of acid, resulted in similar or slightly higher absorbancies but often produced cloudy extracts. The use of phosphoric, acetic, hydrochloric, or nitric acids (0.05 N) as the aqueous phase and in the dye preparation resulted in yields 98.3%, 85.2%, 66.0%, and 14.9%, respectively, of the color yield (OD/μmole of sulfatide) that was obtained with sulfuric acid in the standard assay procedure. Blank solutions prepared from phosphoric and acetic acids were the same as those from sulfuric acid, but those from hydrochloric and nitric acids were blue.

Color production by chloride and nitrate ions in the standard assay procedure was examined by measurement of the color produced by KCl and KNO_3 over a range from 10 to 300 μmoles. The color yield (OD/μmole of chloride or nitrate) was 0.0028 for chloride and 0.0095 for nitrate. Compared to the color produced by sulfatide (27.5 OD units/μmole), the color yield from KCl and KNO_3 was quite low. The spectra of the colored complexes produced by these ions were similar to that from sulfatide; the absorption maximum for chloride was at 640 mμ, and that for nitrate at 645 mμ. Although KCl was used in these studies during the Folch washing of tissue extracts, no color was observed in blank solutions carried through these procedures.

Specificity of the Method

Reactivity of Sulfolipids. The color yields (calculated as OD/μmole of compound) for several sulfolipids and related compounds in the azure A procedure are given in Table 1. The long-chain and lipid sulfates, as well as tetradecyl sulfonate, produced similar and relatively high yields of color as compared to octyl and hexyl sulfates. The absorption curves of the complexes formed by all of these materials were similar in shape although the absorption maxima differed slightly; those of the steroid sulfates and of the fatty alcohol sulfates were at 640 mμ, that of the sulfonate at 637 mμ, and that of sulfatide at 647 mμ. All of the data in Table 1 refer to readings obtained at 645 mμ. No color was produced by estriol trisulfate over a tested range from 0.005 to 0.5 μmole. When a solution of the sulfatide obtained from Dr. Stoffyn was analyzed by the azure A colorimetric procedure, with the Supelco product as standard, the sulfolipid concentration was 0.589 ± 0.013 (n = 5) μmole/ml, a value very similar to that obtained by sulfate and galactose analyses of this preparation, as described above.

Color Production by Other Lipids. The ability of some lipids other than the sulfolipids to produce color in the standard azure A assay was investigated with the following results (the range assayed in μmoles is given in parentheses). No color was produced by tripalmitin (0.01–1.0), cholesterol (0.01–2.0), ceramide (Tipton preparation, 0.1–2.0), ceramide (hydroxy FA, 0.1–1.0), ceramide (nonhydroxy FA, 0.005–1.0), cerasine (0.1–1.0), or lactosyl sphingosine (0.005–1.0). Less than 4% of the color yield produced by equimolar amounts of sulfatide was observed with the following materials: sphingomyelin (0.1–2.0), DL-phosphatidyl ethanolamine (0.01–2.0), DL-phosphatidyl choline (0.01–1.0), phosphatidic acid (0.01–1.0), sphingosine (Tipton preparation, 0.005–2.0), sphingosine (Ciba, 0.10–1.0), sphingosine (Upjohn, 0.1), dihydrosphingosine (0.1), sphingosine sulfate (0.01–2.0), psychosine (0.025–2.0), and cytolipin H (0.1–0.5). Less than 10% of the color yield obtained from equimolar amounts of sulfatide was observed with the following compounds: phosphatidyl serine (0.01–1.0), phospha-

TABLE 1 COLOR YIELDS OF SOME SULFUR-CONTAINING LIPIDS AND RELATED COMPOUNDS

Compound	Color Yield*
	OD units/μmole
Hexyl sulfate†	1.70
Octyl sulfate†	10.7
Decyl sulfate†	23.8
Dodecyl sulfate†	29.5
Tetradecyl sulfate†	31.0
Tetradecyl sulfonate†	29.5
Cholesteryl sulfate†	31.2
Estrone-3-sulfate‡	24.9
Sulfatide§	27.5

* Absorbances (at 645 mμ) of the colored complexes formed with azure A and extracted into C–M.
† Sodium salts.
‡ Potassium salt.
§ The compound obtained from Supelco.

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tidyl inositol (0.01–2.0) and ceramide tetrasaccharide (0.025). Gangliosides, in the range 0.25–0.5 mg, produced 0.16 OD units/mg. Cardiolipin, tested over the range 0.016–0.16 mg, had a color yield of 6.07 OD units/mg. Calculated on the basis of stearic acid as the fatty acid constituent, the color yield is 8.77 OD units/μmole, about 30% of the color yield produced by equimolar amounts of sulfatide. The color yield from phosphatidic acid was 3.7% of that from sulfatide. Since several spots were observed on TLC of some of the commercial preparations that were assayed, the contribution of color due to the presence of traces of reactive impurities cannot be ruled out.

Reactivity of Nonlipid Sulfur-Containing Compounds. Most nonlipid sulfur-containing compounds can be eliminated by extraction (19) and column precedures (29), but their presence in lipid extracts would in any case not interfere with the colorimetric assay for sulfatides. This conclusion is based on the following observations. When tested in the standard assay procedure over a range from 0.01 to 2.0 μmoles the following compounds produced no color: adenine sulfate, L-cysteic acid, L-cysteine, L-cystine, *S*-ethyl L-cysteine, DL-methionine, methionine sulfoxide, D-glucose-3-*O*-sulfate, and D-glucose-6-*O*-sulfate. Several sulfated polymers were also examined for their ability to contribute color in this assay. No color was produced by the following materials over the range indicated: protamine sulfate (0.01–2.6 mg), chondroitin sulfate (0.01–2.6 mg), heparin (0.003–0.56 mg), and hyaluronic acid sulfate (0.013–2.6 mg) (30). Hyaluronic acid (0.003–0.68 mg) also did not produce color.

In addition, these materials did not *interfere* in the analysis of sulfatides. The color yield from sulfatide (0.025 μmole) was unchanged in the presence of L-cysteine, DL-methionine, D-glucose-3-*O*-sulfate, D-glucose-6-*O*-sulfate, and chondroitin sulfate, over the ranges indicated.

Effect of Other Lipids on Sulfatide Analysis. Standard sulfatide (Supelco) was assayed in the presence of large amounts of a variety of purified lipids. The results of this study are seen in Table 2. At levels up to 10 times the concentration of sulfatide a variety of phospholipids and several glycosphingolipids did not affect the color yield from the sulfatide. At 100-fold concentrations, the phospholipids caused considerable decrease in absorbancy. Cardiolipin, the most reactive of the nonsulfolipids that were tested, produced some interference when present at 10 times the concentration of sulfatides. Cerebrosides at 10 or 100 times the concentration of sulfatide did not interfere in the assay. Although sphingosine, psychosine, and lactosyl sphingosine produced little or no color with azure A, these compounds markedly inhibited color formation by sulfatide. The inhibition by sphingosine was observed with several different preparations of this material. Dihydrosphingosine had a similar effect. The ability of this assay to detect sulfatides in the presence of up to 4-fold higher concentrations of several sphingolipids is seen in Fig. 3. The marked inhibition by sphingosine is diminished as sugars are added onto the base (as in psychosine and lactosyl sphingosine), and is completely eliminated when the amino group is acylated (as in the ceramides) or when both the *N*-acyl group and the sugars are present (as in cerebroside and cytolipin H). Cytolipin H did cause some inhibition at a concentration of 50 times that of sulfatide (Table 2), but the presence of about 3% of free sphingosine in this preparation could have produced the effect noted.

Sphingosine also inhibited color formation by cardiolipin. In the presence of 0.05 μmole of sphingosine there was only 29% recovery of the color formed by 0.028 μmole of cardiolipin. Under similar conditions 39% of the color from sulfatide was recovered.

Rouser et al. (27) utilized 2,2-dimethoxypropane as a drying agent in solvents during Florisil chromatography. This material, tested in the range 40–800 μmoles, neither produced color nor interfered in its formation by sulfatides.

TABLE 2 COLOR YIELD FROM SULFATIDE IN THE PRESENCE OF OTHER LIPIDS*

Second Lipid Present	% of Color with Sulfatide Alone	
	10-fold excess	100-fold excess
Sphingomyelin	97.0	70.5
Phosphatidyl ethanolamine	98.1	55.5
Phosphatidyl choline	97.4	70.5
Phosphatidyl serine	91.6	67.1
Phosphatidyl inositol	99.4	86.2
Phosphatidic acid	103.0	74.2
Tripalmitin	103.0	108.0
Cholesterol	102.0	105.0
Sphingosine†	27.0	13.4
Sphingosine (*threo-trans*)‡	27.5	5.09
Dihydrosphingosine	33.0	—
Ceramide†	87.3	30.6
Ceramide (hydroxy FA)	102.0	—
Ceramide (nonhydroxy FA)	98.6	98.8
Psychosine	75.6	42.1
Cerasine	98.2	94.4
Lactosyl sphingosine	63.9	22.1
	Amount Present (μmole)	*% Color Yield*
Cytolipin H	0.10	98.1
"	0.50	69.0
Ceramide tetrasaccharide	0.025	100.0
Gangliosides	0.1 mg	100.0
Cardiolipin§	0.011	101.0
"	0.11	89.0

* Sulfatide, 0.01 μmole, was assayed by the azure A colorimetric procedure described in the text, in the presence of 0.1 and 1.0 μmole of the compounds, except where indicated.

† Prepared according to the procedure of Tipton (18).

‡ The compound obtained from Ciba. The preparation obtained from Upjohn gave similar results.

§ Calculated on the basis of stearic acid as the fatty acid constituent.

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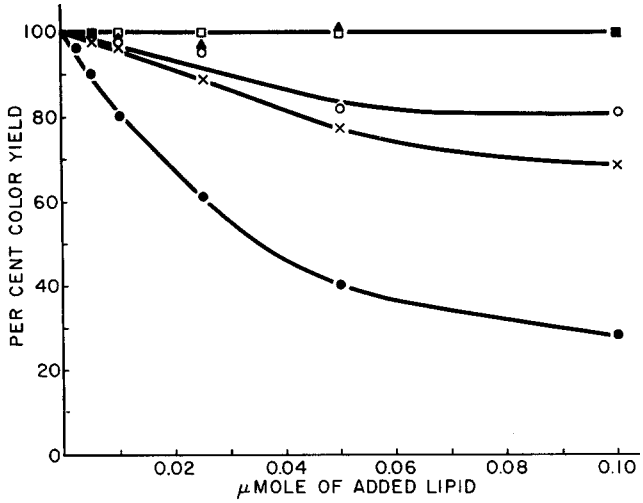

FIG. 3. Color yield (%) of sulfatide in the presence of other sphingolipids. The azure A colorimetric assay was performed on mixtures composed of 0.025 μmole of sulfatide and the indicated amounts of sphingosine (●), psychosine (X), cerasine (▲), lactosyl sphingosine (○), cytolipin H and ceramide (□). (Essentially the same values were obtained for ceramide and cytolipin H.)

Analysis of Tissue Extracts for Lipid Sulfate

This colorimetric assay has a high degree of accuracy, precision, and specificity for sulfatides (except for some of the phospholipids noted previously) in artificial mixtures of lipids. Its usefulness in determining the sulfolipid content of tissue extracts was next examined. Lipids were isolated from rabbit brain tissue as described under Methods. Lipid sulfate was determined in the crude extract and in Folch-washed and Florisil C–M 2:1 fractions by the azure A colorimetric procedure, and total sulfate was measured by the turbidimetric procedure after sealed tube hydrolysis. The data presented in Table 3 show reasonable agreement between the two procedures; there is no way of knowing which method gives the more nearly accurate results. The colorimetric values are lower at each stage of purification; however, the differences are no greater than those observed by Davison and Gregson (3) between values obtained by the turbidimetric and chloranilate methods.

The spectra of the azure A complex obtained from the crude extract and from Folch-washed and Florisil C–M 2:1 fractions from rabbit brain white matter are essentially identical with that of standard sulfatide.

Radioactivity Studies

Additional evidence for the correlation between colorimetric yield and sulfolipid content in extracts of tissue was obtained from a study of the incorporation of ^{35}S into the lipids of brain white matter of adult rabbits. After an injection of $Na_2{}^{35}SO_4$ and subsequent extraction and analysis, the following data were obtained. The Folch-washed fraction contained 7440 cpm/g (wet wt) of tissue, and, on the basis of the azure A assay, 12.2 μmoles of sulfatide per g (wet wt) of tissue. After Florisil chromatography, the C–M 2:1 fraction contained 6360 cpm/g (wet wt) of tissue (a recovery of 85.5%), and 10.6 μmoles of sulfolipid per g (wet wt) of tissue (a recovery of 87.0%). All of the radioactivity of these two extracts migrated with standard sulfatide in solvent systems A and B. According to this evidence, the specific activity of sulfatide was 610 cpm/μmole in the Folch-washed extract and 600 cpm/μmole in the Florisil C–M 2:1 fraction. There was thus a good correlation between lipid-^{35}S (sulfolipid) and colorimetric analyses.

TABLE 3 ANALYSES OF EXTRACTS OF RABBIT BRAIN WHITE MATTER FOR LIPID SULFATE

	Lipid Sulfate	
	Colorimetric*	Turbidimetric†
	μmoles/ml extract	
Crude extract	0.520 ± 0.023 (6)‡	0.62 ± 0.02 (2)
Folch-washed extract	1.38 ± 0.030 (5)	2.02
Florisil C–M 2:1 fraction	1.23 ± 0.058 (8)	1.57 ± 0.05 (2)

* The azure A colorimetric assay as described in the text.
† Analysis after sealed tube hydrolysis according to the method of Green and Robinson (5).
‡ Mean ± SEM (n in parentheses).

Recovery of Sulfatide Added to Tissue Extracts

To various amounts of tissue extracts, selected so that their endogenous yields of color covered the range from about 0.2 to 0.8 OD units, were added the amounts of sulfatide indicated in Table 4. The mixtures were then assayed. Essentially quantitative recovery of added sulfatide was obtained in extracts of brain white matter at all stages of purification. Since gray matter has a much lower content of sulfatide, proportionately larger amounts of extract were required for analysis. This increased the possibility of introducing inhibitors of color formation. Good recoveries of sulfatide were observed with lower levels of the crude extract, while diminished recovery was seen at the higher level. A similar trend was observed in the Folch-washed extracts. At the stage of purification represented by the Florisil C–M 2:1 fraction, which contained primarily cerebrosides and sulfatides, essentially quantitative recovery of added sulfatide was obtained.

Analysis of Sulfolipids After TLC

Although the azure A colorimetric assay has a high degree of specificity it cannot distinguish between the various reactive sulfolipids. In addition to sulfatide, the steroid sulfates (and probably, the dihexose sulfatides) are active. Cholesteryl sulfate has been found in bovine adrenal glands (31), and recently in various human tissues (23). This steroid sulfate and sulfatide have similar solubility properties and would be isolated together by

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TABLE 4 RECOVERY OF SULFATIDE ADDED TO EXTRACTS OF BRAIN TISSUE*

	Endogenous Sulfolipid†	Sulfatide Added	Recovery of Added Sulfatide
	mμmoles		%
Brain white matter			
Crude extract	11	7	103
	11	10	102
	11	15	101
	21	7	104
Folch-washed extract	11	7	103
	11	10	111
	11	15	108
	28	7	103
Florisil C–M 2:1 fraction	17	7	98.6
	17	10	95.0
	17	15	99.4
	23	7	103
Brain gray matter			
Crude extract	5.7	7	97.3
	5.7	10	91.1
	5.7	15	100
	15	7	91.5
	18	7	84.3
Folch-washed extract	10	7	88.6
	10	10	91.0
	10	15	90.0
	13	7	90.0
	17	7	82.9
Florisil C–M 2:1 fraction	10	7	104
	10	10	103
	10	20	97.0
	20	7	94.4

* All analyses refer to the azure A colorimetric assay with the sulfatide from Supelco as the internal standard.

† Amounts of endogenous sulfolipid, in mμmoles/mg of lipid, were: in the crude extract, Folch-washed extract, and Florisil C–M 2:1 fraction of white matter, respectively 71, 79, and 138; and in corresponding fractions from gray matter 29, 29, and 160.

the procedures followed here. Moser, Moser, and Orr (23) have demonstrated, however, that these two compounds can be separated on thin-layer plates if solvent system B is used. 5–25 mμmoles of sulfatide and of cholesteryl sulfate were applied as spots to thin-layer plates. After chromatography, the lipids were located by 1 min exposure to iodine vapor. When the iodine color had disappeared the reactive areas were scraped off the plate into screw-cap tubes and the colorimetric assay was carried out as indicated previously. Although the color yield for sulfatide was only 70% of that expected, the standard curve was linear and reproducible. The color yield was higher (92%) for cholesteryl sulfate and the curve was also linear. Mixtures of sulfatide and cholesteryl sulfate could be successfully assayed by the two-stage procedure, provided that the standard curves obtained *after* the TLC procedure were used.

The possibility that low color yield was due to the presence of silica gel was investigated as follows. Sulfatide was assayed on various amounts of gel that had been impregnated with sulfatide (25 mμmoles). Over 90% yield of sulfatide was obtained in the presence of 2–22 mg of gel. Lower yields were obtained with larger amounts of gel: 45 mg, 87%; 66 mg, 64%; 111 mg, 56%. Since only about 25 mg of gel was obtained from the sulfatide areas after chromatography, interference by the gel is probably not the sole cause of the lower recoveries that were observed.

DISCUSSION

In those tissues in which the sole or predominant class of sulfolipid has been shown to be the sulfatides, such as brain white matter, the colorimetric assay described in this report provides a rapid and sensitive method for the quantitative determination of this important class of compounds.

The low recoveries of sulfatide that were encountered when sulfatide was added to relatively large amounts of crude and Folch-washed extracts of brain gray matter were corrected by the use of Florisil. The reason for the low recovery is not known. Neither free sphingosine nor *N*-deacylated glycosphingolipids (which interfere with the color production) have been observed in mammalian tissues. Furthermore, Rouser et al. (27) have shown that phospholipids, and other lipids, are present in brain at less than 10 times the concentration of sulfatides, and at such concentrations these lipids did not affect the quantitative assay of sulfatide in this study.

Wells and Dittmer have characterized and determined cardiolipin in brain tissue (32). If one can extrapolate from their data, obtained for the brains for young rats, to the adult rabbit used here, it can be estimated that cardiolipin would be present at less than $^1/_{10}$th the concentration of sulfatide. This consideration, together with the observation that sulfatide produces about three times the amount of color (per mole) in the azure A assay as does cardiolipin, suggest that cardiolipin would have contributed little color in assays performed on the crude and Folch-washed extracts. These considerations may apply also to the polyphosphoinositides (33) which, however, were not evaluated in this study. Since some phospholipids do contribute color, it seems desirable to use the colorimetric method only after phospholipids have been removed. Florisil chromatography seems to be a suitable method of purification, since sulfatide added to the Florisil C–M 2:1 fraction is quantitatively recovered, although this precaution is apparently not necessary for lipid extracts from brain white matter.

The major class of glycolipids eluted with sulfatides from Florisil consists of cerebrosides. The observation that the cerebrosides, even at 100 times the concentration of sulfatides, have no effect on the assay supports the suggestion that this stage of purification is adequate for reliable analysis.

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Since the chloride and nitrate salts of azure A are extractable into C–M solutions, perhaps chloride or nitrate should not be used during the Folch washing procedure, even though blanks carried through this procedure have been colorless. Studies in this laboratory (E. L. Kean, unpublished) have shown that 0.1 M Na_2SO_4 is preferable to water for retention of sulfatide in the lower phase during the Folch washing procedure.

The solubility of colored salts of azure A in the chloroform-rich phase depends to a large extent upon the lipoidal or nonpolar nature of the anionic constituent of the salt. Thus, a marked decrease in color yield was observed in the fatty alcohol sulfates when the chain length was less than 10. Although the color yields from cholesteryl sulfate and from estrone-3-sulfate were high, the more polar steroid sulfate, estriol trisulfate, gave no color. Similarly, the chloride and nitrate complexes of azure A are extractable by C–M 1:1 only to a small extent.

Comparison of the different interfering substances also reflects the importance of the polarity of anionic groups, which determines the solubility of the azure A salt in C–M. Thus, the ability of *N*-deacylated derivatives of sphingosine to inhibit color development by sulfatide decreases as their polarity increases. For example (as seen in Fig. 3) the presence of 0.1 μmole of psychosine and lactosyl sphingosine resulted in 43% and 25%, respectively, of the inhibition observed with free sphingosine. The mechanism by which sphingosine inhibits the formation of the colored complex between azure A and sulfatide probably involves competition between the two cationic substances for the anionic sulfolipid. Further studies on the mechanism are in progress.

Note Added in Proof. Triphosphoinositide (TPI, sodium salt, obtained from ox brain) was generously supplied by Dr. C. E. Ballou, University of California at Berkeley. This compound, when tested in the azure A assay over the range 0.01–0.05 μmole (molecular weight was calculated on the basis of stearic acid as the fatty acid constituent), had a color yield of 6.40 OD units/μmole; i.e., about 23% of the color produced by equimolar amounts of sulfatide. TPI had no effect on the color produced by 0.01 μmole of sulfatide when present in equimolar amount, although a decrease (23%) in the color yield from sulfatide was observed when the latter was assayed in the presence of 10 times higher concentration of TPI. As with sulfatide and cardiolipin, sphingosine also inhibited color development by TPI.

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