Only by separating what is known from what is not known according to some tentative overall scheme is it possible to design experiments applicable to the solution of this extremely complex interrelationship. Since relatively few of the myriad biological variables have been considered herein, the conclusions may be of only academic interest. Any experimental condition or dietary alteration which in any way changes membrane permeability (exposure to ionizing radiation, hyper-or hypoxia, various toxic substances such as carbon tetrachloride or certain organo-phosphorus compounds, choline deficiency, various dietary trace metals or necrosis of unspecified origin) might effect lipid peroxidation *in vivo*.

ACKNOWLEDGMENTS

The generous support of Dr. M. K. Horwitt and the assistance of Judith Krishnan, Ruth C. Nelson, M. Jane Morton, and E. M. Harmon are gratefully acknowledged.

This investigation was supported in part by Illinois Mental Health Fund and Public Health Service Research Grant No. AM-07184 from the National Institute of Arthritic and Metabolic Diseases.

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[Received November 19, 1964—Accepted June 11, 1965]

Analytical Separation of Nonlipid Water Soluble Substances and Gangliosides from Other Lipids by Dextran Gel Column Chromatography

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Abstract

A column chromatographic procedure is reported utilizing a dextran gel (Sephadex) for the complete separation of the major lipid classes from water-soluble nonlipids. Lipids other than gangliosides are eluted first with chloroform/methanol 19/1 saturated with water, gangliosides with chloroform/methanol/water containing acetic acid, and water-soluble nonlipids with methanol/water 1/1. Results for adult human whole brain, grey and white matter, and normal infant whole brain lipids are presented. With beef brain lipid as sample the ganglioside fraction is essentially pure, but with human brain lipid samples only about 70% of the second fraction is ganglioside. All ganglioside and water soluble nonlipid of a human spleen chloroform/methanol extract was separated from lipids with the procedure. Control studies with $P^{32}O_4\equiv$ and C_{14} labelled glucose showed that all counts were present in fraction 3. Similar studies with C_{14} labelled amino acids (glycine, serine, alanine, phenylalanine) showed that only phenylalanine counts were eluted in fraction 2 along with the gangliosides. The procedure was applied for removal of large amounts of ammonium acetate from DEAE cellulose column fractions and for complete removal of adsorbent and salts from lipids eluted from thin-layer chromatograms. After passage through the dextran gel columns, lipids eluted from thin-layer chromatograms were found to give infrared spectra identical to those of pure samples obtained by other procedures.

Introduction

WATER-SOLUBLE, NONLIPID substances (sugars, amino acids, and inorganic salts, etc.) are invariably extracted from tissues along with lipids. It is necessary to remove nonlipids from lipids for determination of total lipid by weighing and to prevent contamination of column chromatographic fractions with nonlipids. A convenient, rapid, quantitative means is also desirable for removal of salts introduced into lipid preparations by various laboratory procedures. Gangliosides, the complex group of water soluble lipids, occur in some organs (particularly brain) and present an additional problem for it is desirable to isolate gangliosides free of other lipids and nonlipids for quantitative determination of total gangliosides.

Dialysis, solvent partition, cellulose column chromatography, and DEAE cellulose column chromatography have all been used for separation of lipids from nonlipids (4-8). The recent report of Wells

and Dittmer (9) suggested that the cross-linked dextran gel, Sephadex, would be useful for the column chromatographic separation of lipids from nonlipid water-soluble substances. The procedure of Wells and Dittmer did not give quantitative separation of brain lipid in our hands. Gangliosides were eluted partly with other lipids and partly with water soluble nonlipids, and some nonlipid was eluted with lipids. The characteristics of the dextran gel were investigated further.

This report describes a quantitative procedure for separation of water soluble nonlipids from lipids. With the procedure gangliosides are quantitatively eluted as a separate fraction.

Materials and Methods

Solvents and Nitrogen

Reagent grade solvents were redistilled from glass to remove nonvolatile solids and prepurified nitrogen (less than 7 ppm oxygen) was used for all evaporations and in general laboratory operations to create an inert atmosphere. All solvent mixtures are expressed as volume/volume ratios unless otherwise specified. All water-saturated solvents were mixed at 25 ± 1C. Faintly turbid mixtures can be used as washing and eluting solvents or the excess of water can be removed in a separatory funnel equipped with a Teflon stopcock.

Washing of Sephadex

A 100-g portion of Sephadex G-25 (coarse, beaded; obtained from Pharmacia Fine Chemicals Inc., N.Y.) was placed on a coarse grade sintered-glass filter. Distilled water (2 liters) was added, mixed with the gel with gentle stirring and allowed to filter under gravity. The bed was then washed with 2 liters of acetone in the same manner. The water-acetone wash cycle was repeated for a total of three cycles. The bed was then dried with two additional one-liter portions of acetone, dried for 30 min on the filter under suction from a water pump, spread over filter paper and air-dried overnight in an area free from acid and other vapors.

Solvent Equilibration and Degassing of Gel

Air-dried gel (50 g) was placed in a 1-liter suction flask, 200 ml of methanol/water 1/1 added, the flask covered with aluminum foil, and allowed to stand overnight for equilibration. Dissolved gases were then removed from the solvent-equilibrated gel under suction from a vacuum pump with swirling (to prevent bumping) for 30 to 60 sec.

Column Preparation for Tissue Chloroform/Methanol Extracts

A chromatography tube approximately 2.5 cm (I. D.) and 40 cm long fitted with a Teflon stopcock and a 1-liter reservoir for solvent was used for brain lipid separations. A glass-wool plug moistened with methanol/water 1/1 was used to retain the gel. The gel slurry in methanol/water 1/1 was poured into the tube, the walls of the tube washed with methanol/water 1/1, and the gel bed washed with 500 ml of the same solvent at a gravity flow rate of 5–6 ml/min. The wash solvent should be mixed and allowed to come to room temperature before use to avoid bubble formation. The final column height was adjusted to 30 cm by aspiration of excess gel. Dry, moderately fine grained, reagent grade sand (previously washed with methanol/water 1/1) was poured to a height of about 1 1/2 in. with 2–3 in. of solvent still remaining over the bed. The sand was stirred with a glass rod to insure that the top of the bed was even but care was taken not to stir into the gel bed. The sand prevents floating of the gel when chloroform-containing solvent mixtures are applied and prevents disturbance of the bed during solvent additions.

Careful washing of the gel column before sample application is necessary to prevent appearance of "fines" in the methanol/water 1/1 fraction and was accomplished as follows. The methanol/water 1/1 solvent used for packing the column was allowed to pass completely into the sand layer and 500 ml of chloroform/methanol 19/1 saturated with water (5 ml water per liter) added. The solvent was passed through the bed at a flow rate of 5–6 ml/min and was followed by 1 liter of chloroform/methanol/acetic acid/water mixture (prepared by mixing 5 parts of chloroform/methanol 19/1 with one part of glacial acetic acid and addition of 25 ml of water per liter), 1.5 liters of methanol/water 1/1, 500 ml of chloroform/methanol 19/1 saturated with water (5 ml water per liter), one liter of the chloroform/methanol/acetic acid/water mixture above, 1 liter of methanol/water 1/1, and finally 500 ml chloroform/methanol 19/1 saturated with water. Wash solvents can be passed through the bed at 5–6 ml per minute during working hours, the rate being adjusted downward at the end of the day to prevent the column from running dry overnight.

The appearance of irregular opaque areas in the column can be ignored since column performance is not affected to any appreciable extent. When the column has been equilibrated for 24–72 hr in methanol/water 1/1 before replacement with chloroform/methanol 19/1 saturated with water and application of the sample, the uneven appearance of the column is largely avoided. Each solvent change results in the appearance for a time of a two-phase system in the effluent (at the solvent front). The solvent mixtures properly saturated with water do not produce significant changes in column height or solvent flow rates.

Sample Application

Chloroform/methanol extracted lipid (100–350 mg) of brain and other organs containing water soluble nonlipids was applied in about 5 ml of chloroform/methanol 19/1 saturated with water (5 ml water/liter) and lipid and any suspended solids rinsed in with the same solvent. Samples containing large amounts of suspended solids can be transferred conveniently to the column with a 5 or 10 ml blowout type pipette with the opening of the tip enlarged to prevent blockage. Only about 1 ml of solvent containing sample should be drawn into the transfer pipette each time to lessen the area of contact and the volume of solvent required for complete transfer. The transfer pipette is rinsed inside and out with solvent delivered from a 1 ml pipette after transfer of sample is complete. A glass wool plug (washed with chloroform/methanol/water) is added after sample application to further protect the column surface and prevent sand from moving up into the solvent reservoir.

Elution of Column

After sample application, bulk fractions were collected at a flow rate of 3 ml/min. Four eluting solvents were used to give three fractions. The solvents and volumes collected were:

1) 500 ml of chloroform/methanol 19/1 saturated with water (5 ml/liter).
2) 1000 ml of a mixture of 5 parts of chloroform/methanol 19/1 (850 ml) and 1 part of glacial acid (170 ml) to which is added 25 ml of water (mix several times in a separatory funnel with a Teflon stopcock and use clear lower phase).
3) 500 ml of a mixture of 5 parts of chloroform/methanol 9/1 (425 ml) and 1 part glacial acetic acid (85 ml) to which is added 21 ml of water (mix several times in a separatory funnel with a Teflon stopcock and use clear lower phase).
4) 1000 ml of methanol/water 1/1.

The effluents collected after application of solvents 2 and 3 were usually pooled and evaporated together as a single fraction.

Columns are preferably allowed to stand in methanol/water 1/1 for about 48 hr before reuse, 500 ml chloroform/methanol 19/1 saturated with water being passed through the bed just prior to application of another sample.

Evaporation and Weighing of Fractions

Handling of fractions followed the procedures described by Rouser et al. (7). Each fraction was evaporated below 10C in a rotary (flash) evaporator flushed with pure nitrogen. Two cold traps in series cooled with a mixture of methyl cellosolve-dry ice were used and reduced pressure was provided by a vacuum pump. Solids were transferred with small volumes of chloroform/methanol 2/1 (fractions 1 and 2) or methanol/water 4/1 (fraction 3) to 50 ml filtering flasks and solvent removed on a vacuum rack (7) under a stream of nitrogen provided by insertion of a rubber stopped fitted with a capillary tube and connection to a vacuum pump through the side-arm of the flask. The evaporations were carried out at low temperature and the flasks usually became caked with ice that melted when all solvent was removed. The fractions in the small flasks were dried to constant weight over KOH in a vacuum desiccator (under vacuum). Weighing was accomplished with a semi-micro balance with reproducibility of $\pm$ 0.05 mg.

Thin-Layer Chromatography (TLC)

One-dimensional TLC was used routinely to examine all fractions for lipid components (other than gangliosides). TLC was carried out with the silicic acid-magnesium silicate adsorbent of Rouser et al. (10) prepared by mixing 9 parts of Silica Gel Plain (Warner-Chilcott Laboratories Instruments Division, Richmond, Calif.) with 1 part of finely powdered magnesium silicate (Allegheny Industrial Chemical Co., P. O. Box 786, Butler, N. J.). The mixture was heated at 120C for 6 hr, cooled in the absence of air, ground and mixed on a ball mill, and kept in a tightly stoppered bottle. Glass plates 8 in. square were spread with a slurry of 20 g of adsorbent in 65 ml of water with a Desaga fixed-distance (250μ) spreader. The plates were heat activated for 20 min at 120C, cooled for 30 min, spotted with sample applied from a microliter syringe, and developed immediately with the desired solvent.

Solvents for one-dimensional ascending TLC were the various chloroform/methanol/water mixtures used previously (8,10), mixtures of chloroform/methanol/28% (by weight) aqueous ammonia (65/35/5, 65/25/4) (8); and mixtures of chloroform/acetone/methanol/acetic acid/water (5/2/1/1/0.5, 5/2/1.5/1.5/1) (8). Two-dimensional TLC was carried out with the general technique described previously (8, 10) by development with chloroform/methanol/water or chloroform/methanol/aqueous ammonia mixtures, air-drying for no more than 10 min, and development in the second dimension with butanol/acetic acid/water 60/20/20 or one of the chloroform/acetone/methanol/acetic acid/water mixtures (8). All TLC was carried out in chambers lined on all sides with solvent saturated paper. Acidic solvents were always used for development in the second dimension to avoid destructive changes of lipids brought about by acid during drying of chromatograms.

The individual ganglioside components in lipid mixtures were separated by one dimensional ascending TLC using 1-propanol/water 70/30 as solvent in a chamber lined with solvent saturated paper.

The sulfuric acid-potassium dichromate spray reagent of Blank et al. (11), as modified by Rouser et al. (10), was used as a general means for visualizing spots from all lipids. After light spraying with the reagent as a fine mist (10), spots were developed at 180C for 30–60 min in a forced-draft oven and chromatograms photographed with a Polaroid camera (12).

Specific spray reagents used for TLC were ninhydrin (1 mg/ml in chloroform/methanol/water 4/4/1 mixed with one-quarter volume of lutidine just before use) followed by heating 3–5 min at 120C, the phosphate spray of Dittmer and Lester (13), and the resorcinol spray for neuraminic acid (ganglioside) of Svennerholm (14).

A specific α-naphthol spray for glycolipids (cerebrosides, sulfatides, gangliosides) was developed. A 0.5% solution of α-naphthol (recrystallized from *hexane/chloroform*) in methanol/water 1/1 was sprayed as a fine mist until the chromatogram was damp. The plate was air-dried and sprayed very lightly with a fine spray of 95% (by weight) sulfuric acid. Color development was achieved by either of two procedures. Heating the chromatogram at 120C until maximum color development takes place results in production of bluish-purple spots for glycolipids and yellow spots for other polar lipid classes (cholesterol gives a grey-red spot). If the chromatogram is covered with a glass plate to prevent drying and is heated at 120C, glycolipids give somewhat stronger bluish-purple spots and phosphatidyl ethanolamine gives a yellow spot. Other brain lipids do not give spots with this means of color development.

Paper Chromatographic Methods

Detection of small amounts of nonlipids in lipid fractions was accomplished by ascending paper chromatography using Whatman 3MM paper and the 1-butanol/acetic acid/water (4/1/5, upper phase) solvent in unlined chambers, as previously described (12). For this purpose solvent was allowed to travel at least 12 in. and as much as 2 mg of lipid applied to one spot. Such large loads are possible because lipids other than gangliosides migrate with or near to the solvent front.

The same general chromatography conditions, but with paper only 8 in. long and solvent migration of 6–8 in. in unlined chambers, was used routinely for detection of gangliosides in fractions. The different gangliosides are not separated to any extent with this solvent system and it is thus convenient for detection of even traces of all types of gangliosides in fractions.

Localization of lipids including gangliosides was ac-

TABLE I

Sample	Sample wt. (mg)	Fraction wts. (mg) 1	2	3	Recovery %
Conditioned columns [a]					
Whole adult human brain	244.48	216.30 (88.47%)	8.55 (3.49%)	21.38 (8.74%)	100.7
Adult human brain, grey matter[b]	326.26	249.95 (76.69%)	28.23 (8.65%)	51.39 (16.05%)	101.4
Adult human brain, white matter[b]	269.20	234.14 (86.97%)	11.20 (4.16%)	27.61 (10.25%)	101.4
Whole normal 1 day old infant brain	286.72	208.87 (72.85%)	21.39 (7.46%)	55.28 (19.28%)	99.6
Synthetic mixture [c]	64.90	34.02	6.12	25.03	100.4
Unconditioned column [d]					
Adult human brain, grey matter	268.78	207.33 (77.14%)	24.72 (9.19%)	49.98 (18.58%)	104.9

[a] Columns packed in methanol/water 1/1 and cycled two times through the solvents used in the elution scheme.
[b] Representative samples of whole brain prepared by quantitative aspiration of grey matter from slices of one-half brain. Slices cooled to the point where white matter was solid but grey matter remained fluid and could be aspirated.
[c] Cholesterol, 34.29 mg; beef brain ganglioside, 6.00 mg; water soluble nonlipids, 24.61 mg.
[d] Column packed in methanol/water 1/1, and washed only with chloroform/methanol 19/1 saturated with water before application of sample.

complished by dipping air-dried (overnight) chromatograms into a 0.001% solution of rhodamine 6G, removing excess dye by washing in a pan of water, and viewing while wet under short-wave ultraviolet light. After a short drying time (but when chromatograms were still slightly moist), lipid areas were observed to stand out particularly well as bright yellow spots against a purple background.

Free amino acids were detected by spraying with a solution of ninhydrin in 1-butanol (1 mg/ml) to which one-quarter volume of lutidine (or pyridine) was added immediately before use. Chromatograms were heated at 120C for several minutes for color development. A modification of the alkaline silver nitrate procedure of Trevelyan et al. (15) was used for localization of spots of polyols and sugars after paper chromatography with butanol/acetic acid/water. The chromatogram was partially air-dried (but not dried completely to avoid loss of glycerol or other volatile substances) and dipped into an acetone solution of silver nitrate (0.5 ml saturated aqueous $AgNO_3$ per 100 ml acetone with water (about 1 ml) added dropwise to clarify the solution) and dried completely in air. The paper was then dipped into 0.5 N ethanolic KOH for 1–3 min (maximum color development for lipids such as phosphatidyl inositol requires about 3 min), rinsed in tap water, dipped for about 10 sec in 3% (by weight) aqueous ammonia, rinsed in tap water, and dipped for about 30 sec in 3% sodium thiosulfate. The entire procedure is carried out in enamel or glass trays. With practice, the ammonia and sodium thiosulfate washes can be performed to leave a pale brown background with dark brown or black spots. The spots are stable for prolonged periods when chromatograms are not exposed to light and air. As little as 0.1 μg of inositol and 1 μg of phosphatidyl inositol were detected with the reagent.

Lipid Standards for Paper and Thin Layer Chromatography

The various brain lipid classes were isolated by column chromatography (7). Cardiolipin was isolated from lipids of beef heart mitochondria by DEAE cellulose column chromatography (7). Routine chromatography was accomplished with whole beef brain lipids, mitochondrial lipids, and a commercial soybean phospholipid mixture (Asolectin, Associated Concentrates, Woodside, Long Island, N.Y.) as standards. Brain lipids serve as a convenient sample for placement of lecithin, sphingomyelin, phosphatidyl serine, phosphatidyl ethanolamine, cerebrosides, sulfatides, gangliosides, and cholesterol. Beef heart mitochondrial lipids were convenient for placing diphosphatidyl glycerol (cardiolipin) on chromatograms because of the high content of this lipid and the fact that the only other lipid classes present in large amount are lecithin and phosphatidyl ethanolamine. The commercial soybean phospholipid mixture was used as a convenient sample for placing phosphatidic acid and phosphatidyl inositol on chromatograms since both of these lipid classes are present in relatively large amount in the mixture. Two-dimensional chromatography of the components of beef brain, mitochondrial lipids, and Asolectin have been presented (8,10).

Dextran Gel Columns for Cleanup of Lipids Isolated by Thin-Layer Chromatography

Small samples of lipids eluted from thin-layer chromatograms may contain salts and some finely divided adsorbent. The extraneous materials can be removed completely with a Sephadex column to give a very pure lipid sample with quantitative recovery of the lipid.

A dextran gel column 0.8 x 12.5 cm prepared as described above using 35 ml of each solvent for column conditioning and elution is convenient for removal of salts and adsorbent from lipids isolated by TLC. A chromatography tube (15 cm long) equipped with a 50 ml capacity solvent reservoir bulb and a Teflon stopcock is convenient. The lipid samples are applied in chloroform/methanol 19/1 saturated with water and eluted with 35 ml of the same solvent at a flow rate of 0.5 ml/min. After solvent removal and drying to constant weight in a vacuum desiccator over KOH, the lipid is weighed and infrared examination (6,7) used to demonstrate purity.

Isotope Labelled Compounds and Counting

Radioactive inorganic phosphate (P^{32}) was obtained from Oak Ridge, Tenn., in weak hydrochloric acid solution that was neutralized with 1 N NaOH. C^{14} labelled glucose and amino acids were obtained from Nuclear-Chicago (333 E. Howard Ave., Des Plaines, Ill.). Counting was done in a Tri-Carb scintillation counter with standard PPO-POPOP counting

TABLE II

Sample	Sample wt. (mg)	Isotope	Counts/min applied	Fraction 1 (c/min)	2 (c/min)	3 (c/min)	Recovery %
Whole adult human brain [a] + P^{32}	244.48	$P^{32}O_4$	34,560,000	0	0	19,040,000	55.1 [c]
Synthetic mixture containing C^{14} labelled water solubles [b]	64.90	C^{14}	5,903,000	0	805,000 [d]	5,185,000	101.5

[a] See Table I for weight recoveries.
[b] See Table I for weight recoveries and composition of the mixture. Radioactive amino acids (serine, glycine, alanine, and phenylalanine) and radioactive glucose were added.
[c] Remaining P^{32} was adsorbed on column and glass surfaces.
[d] Counts from C^{14} phenylalanine.

mixtures (3 gm PPO, 100 mg POPOP per liter of toluene). The final counting solution was prepared by mixing 15 parts of the counting mixtures with 1 part of hydroxide of Hyamine.

Results

The data in Table I show the excellent recoveries obtained with several brain samples and a synthetic mixture. Recoveries tend to be slightly high even on properly conditioned columns. When columns are not cycled twice through the elution solvents before sample application (unconditioned columns), recoveries are quite high. Fraction 3 is particularly high with unconditioned columns (see comparison of grey matter samples on conditioned and unconditioned columns, Table I) since "fines" are eluted more extensively with methanol/water 1/1. If an improperly placed glass-wool plug should allow passage of gel into either of the first two fractions, the fractions can be freed of gel after evaporation of solvent by solution in chloroform/methanol 2/1 and filtration through a tight glass-wool plug (solvent washed) in the stem of a small funnel.

Table II illustrates the recoveries of P^{32} and C^{14} added as inorganic phosphate, glucose, and amino acids. Inorganic phosphate appears entirely in fraction 3 as do glucose and the more polar amino acids. Phenylalanine appears in fraction 2.

Figures 1 through 6 illustrate the use of paper and thin-layer chromatography for the demonstration of components of fractions. Illustrations showing both the presence and absence of contaminants in fractions are shown. Figure 1 shows how contamination of fraction 1 with ganglioside can be detected by TLC, and Figure 2 demonstrates the absence of gangliosides from fraction 1 from a Sephadex column. Figure 3 illustrates the detection of TLC of a slight contamination of fraction 2 with sulfatide when the column was not properly prepared prior to application of the sample. Detection of lipid other than ganglioside in fraction 2 by paper chromatography is illustrated in Figure 5 (same samples shown in Figure 3) and Figure 6 shows complete absence of lipids other than gangliosides from fraction 2 of a properly prepared column. Both TLC and paper chromatography are very sensitive means for detection of contamination of fraction 2 with lipids other than gangliosides. The slight contamination illustrated in Figures 3 and 5 represents 1 to 3 μg of lipid/200 μg total lipid or 0.5 to 1.5% of fraction 2 even when chromatography conditions are not ideal. The loss of sulfatide in fraction 2 illustrated in Figures 3 and 5 represents less than 2% of the total sulfatide in the samples. Figure 4 shows ninhydrin positive components in fractions 2 and 3. Most amino acids appear in fraction 3 but a small amount of less polar material can be seen in fraction 2 from human brain samples. This is not seen in fraction 2 with beef brain as sample.

Phosphatidyl ethanolamine is quite unstable and undergoes many decompositions reactions. It was observed that this lipid class is even less stable after separation of lipids from gangliosides and salts by Sephadex column chromatography. A spot produced by decomposition of phosphatidyl ethanolamine can be seen on chromatograms with the proper solvent pairs. The spot (designated as A) is shown in Figures 1 and 2. Production of this artifact is greatest when a small amount of lipid is dried over a large glass surface (as during solvent removal with a rotary

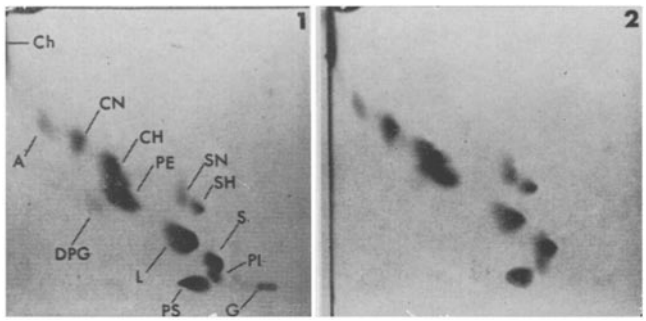

FIG. 1. Two-dimensional TLC of human brain grey matter lipid to illustrate the detection of a trace of ganglioside in fraction 1 from a column that was not prepared properly. Lipid (400 μg) was applied in the lower right hand corner and the chromatogram developed vertically with chloroform/methanol/28% by weight aqueous ammonia (65/35/5), dried in air for 10 min and developed in the horizontal direction with chloroform/acetone/methanol/glacial acetic acid/water 5/2/1/1/0.5. Spots were localized with sulfuric acid-potassium dichromate reagent. Note the presence of gangliosides (at the point of application) in the fraction. Compare with Figure 2 showing the absence of ganglioside in fraction 1 from a column properly washed and equilibrated.

A, artifact formed by decomposition of phosphatidyl ethanolamine; CN, cerebroside with normal fatty acids; CH, cerebroside with hydroxy fatty acids; PE, phosphatidyl ethanolamine; SN, sulfatide with nonhydroxy fatty acids; SH, sulfatide with hydroxy fatty acids; DPG, disphophatidyl glycerol (cardiolipin); L, lecithin; S, sphingomyelin; G, gangliosides; PI, phosphatidyl inositol; PS, phosphatidyl serine; PA, phosphatidic acid.

FIG. 2. Two dimensional TLC of fraction 1 from a Sephadex column, with adult human brain white matter as sample, illustrating the absence of ganglioside and the components present in fraction 1. Lipid (400 μg) was applied in the lower right hand corner. See legend of Figure 1 for chromatography conditions and abbreviations.

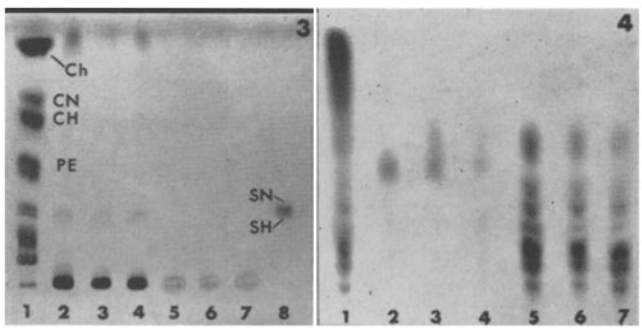

FIG. 3. One dimensional TLC developed with chloroform/methanol/water 65/25/4 (char spray), showing a slight contamination with sulfatide of fraction 2 from an improperly prepared Sephadex column. Applications were: 1) 250 μg whole brain lipid; 2, 3, and 4) 200 μg each of fraction 2 from whole brain, grey matter, and white matter, respectively; 5, 6, and 7) 200 μg each of fraction 3 from whole brain, grey matter, and white matter, respectively; 8) 10 μg of pure sulfatide. Even though contamination of fraction 2 with sulfatide was detected, the contamination was very low (0.5 to 2% of the total fraction 2 or less than 2% of the total sulfatide present in the brain samples as determined by the charring densitometry technique (10)). Compare with paper chromatographic results in Fig. 5 with the same ganglioside fractions.

FIG. 4. One dimensional paper chromatogram developed with 1-butanol/acetic acid/water (4/1/5, upper phase) and sprayed with ninhydrin reagent to show amino acids and other ninhydrin positive substances in fractions 2 and 3 from Sephadex columns. Applications were: 1) 400 μg of whole brain lipid before passage through a Sephadex column showing ninhydrin positive lipid spreading back from the solvent front and amino acids in the lower one-half of the chromatogram; 2, 3, and 4) 100 μg each of fraction 2 from Sephadex column with samples of whole brain, grey matter, and white matter, respectively, showing some ninhydrin positive nonlipid in the fraction (always obtained with human brain samples but not with beef brain); 5, 6, and 7) 100 μg of fraction 3 from the same Sephadex columns, demonstrating that most amino acid appears in fraction 3 (Table II).

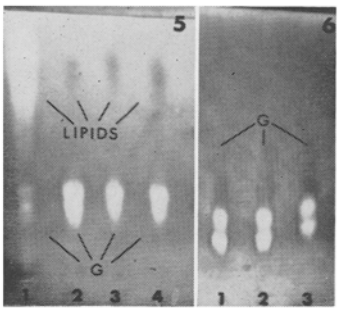

FIG. 5. One dimensional paper chromatogram prepared as described for Figure 4 but stained with rhodamine 6G. The chromatogram illustrates the presence of a trace of sulfatide in the ganglioside fractions from an improperly prepared column (compare Fig. 3). The absence of sulfatide from the ganglioside fraction when all chromatography conditions are proper is shown in Figure 6. Applications were: 1) 250 μg whole brain lipid (showing lipid as a large spot spreading back from the solvent front and ganglioside spots below); 2, 3, and 4) 100 μg each of fraction 2 from whole brain, grey matter, and white matter, respectively, showing a trace of lipid (sulfatide) as a purple spot just behind the solvent front and the bright yellow spots of gangliosides.

FIG. 6. Paper chromatogram prepared as described for Fig. 4 and stained with rhodamine 6G to show complete absence of lipids other than gangliosides from fraction 2 of a properly prepared column.

evaporator), with increase in temperature and with increased exposure to air. The decomposition reaction appears to be related to oxidation at a surface that is accelerated by heat.

A colorimetric assay procedure for gangliosides with α-naphthol and pure beef brain ganglioside as standard was used to show that fraction 2 from the Sephadex columns with beef brain was very pure ganglioside. With human brain, the assay procedure indicated that about 30% of fraction 2 was nonlipid.

Table I shows several interesting features of human brain lipid chloroform/methanol extracts. Adult whole brain contains less ganglioside and water soluble nonlipids than whole immature brain (a one-day-old infant). Adult brain grey matter is, however, very similar to whole infant brain with respect to the relative amounts of the three fractions, although TLC shows the distribution of lipid classes in fraction 1 to be very different for adult brain grey matter and whole infant brain. Although adult brain white matter contains a lower proportion of gangliosides than grey matter, the total amount of gangliosides of brain present in white matter is about the same as that in grey matter because white matter contains much more lipid (16). The values for human brain lipids in Table I are the most accurate thus far presented. The whole brain samples were truly representative of whole brain and were not subject to regional differences since an aliquot was removed for extraction after homogenization of whole brain. Similarly the values for grey and white matter are representative of whole brain and not subject to regional variations resulting from sampling a small portion of brain since the samples of grey and white matter were isolated quantitatively from slices of one-half of a brain specimen (16). Table III presents a useful tabulation of total lipid values of adult and infant brains.

The dextran gel column procedure was used for separation of the lipids of beef brain and human spleen. The ganglioside fraction from beef brain was almost pure in contrast to that prepared from human brain. A relatively large ganglioside fraction (3.3% of the total lipid) was obtained from spleen. Separations were very successful with subcellular particle preparations (nuclei, myelin, mitochondria, microsomes, ribosomes, and nerve ending particles) from beef brain. The procedure for preparation of the subcellular particle utilized centrifugation in solutions of diodrast and sucrose and hence both these substances were present to varying extents in the preparations. Sucrose was present only in fraction 3, but diodrast was eluted in fraction 2.

Samples containing as much as 5 g of ammonium acetate (obtained by elution of DEAE cellulose columns with a chloroform/methanol/ammonium acetate mixture) were applied to the 2.5 x 30 cm columns and lipid separated quantitatively from salt. The dextran gel columns are somewhat more convenient than the multiple evaporation technique used previously for ammonium acetate removal (7) since the column procedure requires less attention and all of the salt is removed relatively rapidly.

Several difficulties are encountered in the preparation of pure lipid samples by TLC. Contaminants are of two types: lipid and other solvent soluble substances taken up by the adsorbent during processing or by exposure of the adsorbent to air, and silicates and other salts from the adsorbent itself. Sephadex columns are useful for removal of silicates and other types of salts from lipids eluted from TLC. We have invariably found that removal of lipid contaminants by solvent wash prior to TLC along with care to prevent introduction of additional contaminants by exposure to air eliminates lipid and other soluble contaminants. The combination of careful washing of adsorbent prior to TLC and Sephadex column chromatography for removal of salt and adsorbent impurities eluted with lipid has given pure preparations of the more stable lipids in almost quantitative yield (98 to 102%).

Discussion

Dialysis in water against water has been used as a means of removal of water soluble nonlipids from lipids, but it is difficult to perform quantitatively. Folch et al. (1) introduced a modified dialysis procedure performed by layering 50 volumes of water over a vessel containing 1 volume of chloroform/methanol 2/1 extract. We have experienced difficulties in making this procedure quantitative. In our hands gangliosides and water soluble nonlipids that appear largely in the upper methanol/water phase are not always removed quantitatively from the lower phase, and some lipid is lost in the upper phase. The large volume of solvent required for dialysis presents major difficulties when even moderately large volumes of chloroform/methanol extract are to be processed.

Folch et al. (2) subsequently described a procedure

TABLE III

Lipid Content of Human Brain

	Av. fresh wt. (gm)	C/M Extract (% fresh wt)	H_2O %	Lipid in C/M extract (%) [a]	Lipid (% fresh wt) [a]	Lipid (% dry wt) [a]	Total Lipid in brain (gm) [a]
Adult whole normal brain [b]	1250	11.9	78.3	88.5	10.5	48.4	131.2
Infant whole normal brain [c]	590	6.2	85.8	74.3	4.6	32.4	27.1

[a] Excluding gangliosides.
[b] Average 5 brains.
[c] One brain only.

for removal of water-soluble nonlipid (along with gangliosides) from chloroform/methanol extracts by washing extracts with aqueous salt solutions. When these procedures were introduced, no satisfactory means were available for demonstration of completeness of removal of water soluble nonlipid from the lower lipid phase and the presence of relatively small amounts of lipid in the upper salt phase. Although widely used, difficulties have been encountered with the Folch procedure. Berman and Gatt (17) found that ganglioside in chloroform/methanol extracts of Tay-Sachs disease brain remained for the most part in the chloroform phase, and Therriault (18) reported the presence of water-soluble nonlipids in the chloroform phase of brain extracts after a Folch aqueous salt solution wash. It is now generally recognized that small amounts of sulfatides and phosphatidyl serine appear in the upper phase with the Folch wash procedure. We have observed that use of this procedure for removal of sucrose from chloroform/methanol extracts of beef heart mitochondria (obtained by centrifugation from sucrose solutions) results in the removal of sucrose, but introduces salt into the lipid phase and leaves the total water soluble nonlipid content almost the same as before washing.

Lea and Rhodes (3) and Smith (4) reported the use of cellulose column chromatography for removal of nonlipids from lipid extracts, and Svennerholm (5) described a cellulose column procedure for incomplete separation of brain extracts into three fractions composed largely of lipids (other than gangliosides), gangliosides, and water-soluble nonlipids. Rouser et al. (6) reported difficulties in reproducibility of the Svennerholm type of cellulose chromatography and in a later report (16) described a modified cellulose column procedure allowing the almost complete separation of lipids (other than gangliosides) from gangliosides and nonlipid water soluble substances. Repeated attempts to obtain quantitative separation of gangliosides from water-soluble nonlipids failed and with the best procedure devised traces of phosphatidyl serine and sulfatide appeared with the ganglioside-water soluble nonlipid fraction.

Rouser et al. (6) reported the separation of water-soluble nonlipids from brain lipids (including gangliosides) on DEAE cellulose columns. Gangliosides were eluted with phosphatidyl serine from DEAE and separated on a silicic acid column. Later, Rouser et al. (7) reported a modified elution scheme for DEAE columns with which water soluble nonlipids were eluted in one fraction (free of lipid) and phosphatidyl serine and gangliosides were eluted separately. The DEAE procedure has provided the only reliable values for total lipid, ganglioside, and water-soluble nonlipids in brain extracts until recently.

The report of Wells and Dittmer (9) indicated that column chromatography with Sephadex would provide a suitable means for separation of water soluble nonlipids from lipids. The procedure in our hands was not satisfactory as a quantitative method. Water solubles are not easily separated from lipids with the solvents of high polarity recommended by these investigators; gangliosides (not studied by Wells and Dittmer) appear in part with the lipids in the first fraction and partly with water-soluble nonlipids. Moreover, the solvent mixtures used in their procedure cause changes in the gel bed volume that make reuse of columns with reproducible performance impossible and "fines" appear in column effluents.

The new dextran gel procedure described above is very reproducible and the columns can be reused repeatedly if not allowed to run dry or if bubble formation from changes in temperature are prevented. The bed does not swell or shrink to any appreciable extent with the solvents used. The procedure gives a complete and wide separation of the other lipid classes from gangliosides as well as gangliosides from most water soluble nonlipids. Traces of the least polar amino acids (phenylalanine and tyrosine) appear with gangliosides. Diodrast introduced in the preparation of subcellular particles of brain was found to be eluted with gangliosides. The procedure is essentially quantitative for each fraction and isotope experiments demonstrate that even minute traces of most water solubles are not eluted with gangliosides. This is in contrast to the results reported by Wells and Dittmer.

When the proportion of methanol or methanol/water in chloroform is increased for elution of lipids, gangliosides are eluted, at least in part, with the other lipids. Attempts to elute pure gangliosides free of non-lipids with chloroform/methanol/water or chloroform/methanol/ammonia mixtures were unsuccessful despite trials with many solvent ratios. Fraction overlap, although sometimes small, was always demonstrable. With the solvent mixture containing acetic acid, a ganglioside fraction free of other lipids is obtained from beef and human brain. Ganglioside is contaminated with traces of less polar amino acids in human brain lipid. In the weakly acidic system the lipid character of gangliosides is increased since ionization of the carboxyl group is decreased. This polarity and solubility change is adequate for separation of gangliosides from most nonlipids. Some gel filtration may take place and increase retention of water soluble nonlipids.

The present procedure is recommended for general application to tissue lipid extracts and is recommended as a substitute for cellulose column chromatography in the procedure of Rouser et al. (16), utilizing cellulose and DEAE columns followed by TLC, for quantitative analysis of components of lipid extracts.

ACKNOWLEDGMENTS

This work was supported in part by U. S. Public Health Service Grants NB-01847-07 from the National Institute of Neurological Diseases and Blindness and CA-03134-09 from the National Cancer Institute. Dr. Akira Yamamoto and Dr. Gene Kritchevsky of the City of Hope Medical Center checked the column procedures for brain and spleen lipids and lipids eluted from thin-layer chromatograms.

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[Received May 6, 1965—Accepted July 8, 1965]