Such a hypothesis could account for the variations in deuterium isotope effect for the enzymatic reaction in the presence and absence of pyridine nucleotide, *i.e.*, there may be less rapid D_2O inactivation of the enzyme in the presence of pyridine nucleotide.

Acknowledgment

The authors are indebted to Dr. Theodore Cohen of the Chemistry Department, University of Pittsburgh, for many helpful discussions concerning the interpretation of the results presented in this paper.

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A Microanalytical Technique for the Quantitative Determination of Twenty-four Classes of Brain Lipids*

Michael A. Wells† and John C. Dittmer‡

ABSTRACT: Selective mild alkaline and acid hydrolyses are used to obtain water-soluble phosphate esters characteristic of the diacyl phosphoglycerides and plasmalogens of brain. These phosphate esters are separated by anion-exchange chromatography and quantitatively assayed. Phospholipids stable to hydrolysis are assayed after fractionation on silicic acid. The gangliosides, neutral lipids, and glycosphinogolipids are measured by specific spectrophotometric determination of characteristic components after an initial solvent fractionation and chromatography on Florisil. Complete analysis of 24 classes of lipids can be carried out on as little as 150 mg of brain tissue. Evidence for the occurrence in rat brain of plasmalogenic acid, choline plasmalogen, inositol plasmalogen, and phosphatidylglycerol is presented. Serine plasmalogen is absent.

No tissue has a more complex lipid compositon than brain and the degree of complexity has only become apparent over the years. It is not surprising then that methods devised in the past to analyze part or all of the different lipids have become obsolete. These methods have ranged from the pioneering attempts of Koch and Koch (1913) who assayed four major classes of lipids through the comprehensive analyses of Brante (1949) which distinguished 10 or 11 classes. More recently and of current value Dawson (1960) and Dawson *et al.* (1962) have developed a system of analysis based on selective hydrolysis procedures and paper chromatography and electrophoresis with which 11 phospholipids of ox and sheep brain have been determined. Also recently, Rouser *et al.* (1963, 1965) have described in general terms analytical procedures based on the fractionation of the intact lipids of brain. Data for 14 lipids of ox brain and 11 of human brain have been reported.

The success of Lester (1963) in quantitatively analyzing the phospholipids of yeast by selective hydrolysis and fractionation of the water-soluble phosphates on anion-exchange columns suggested the possibility of developing a similar technique which, coupled with other specific assays, could be used to analyze all the

* From the Department of Biochemistry, College of Medicine, University of Kentucky, Lexington, Kentucky 40506. *Received August 3, 1966.* This work forms in part a dissertation submitted by M. A. W. to the University of Kentucky in partial fullfillment of the requirements for the Degree of Doctor of Philosophy and was supported by a grant (AM-06008) of the Institute of Arthritis and Metabolic Diseases of the U. S. Public Health Service.

† Predoctoral Fellow, Institute of General Medical Sciences, U. S. Public Health Service. Present address: Department of Biochemistry, University of Washington, Seattle, Wash. 98105.

‡ Present address, Department of Biochemistry, Saint Louis University School of Medicine, Saint Louis, Mo. 63104.

presently known lipids of brain. The result is a method by which 24 different classes of lipids can be analyzed on as little as 150 mg of whole brain tissue.

Experimental Section

Materials. Batyl, chimyl, and selachyl alcohols, cholesterol, cholesterol stearate, digitonin, and L-3-glycerol phosphate dehydrogenase (Calbiochem, Los Angeles); glucosamine and *N*-acetylglucosamine (General Biochemicals, Chagrin Falls, Ohio); *N*-acetylneuraminic acid (Nutritional Biochemicals, Freehold, N. J.); diphosphopyridine nucleotide (Sigma, St. Louis); chlorotrimethylsilane, hexamethyldisilazane, and palmitaldehyde bisulfite (K and K Laboratories, Plainview, N. Y.); *p*-nitrophenylhydrazine (Eastman, Rochester, N. Y); galactose and chloranilic acid (Fisher Scientific, St. Louis); alumina, suitable for chromatographic absorption (Merck and Co., Rahway, N. J.) were all obtained commercially and were either reagent grade or the purest grade available. All of these were used without further purification except that galactose was recrystallized from aqueous ethanol to remove traces of glucose, cholesterol was crystallized from ethanol and *p*-nitrophenylhydrazine from 95% ethanol. The *p*-nitrophenylhydrazone of palmitaldehyde was prepared from the bisulfite addition product and was crystallized from ethanol to a constant melting point of 96.5°. Barium chloranilate was prepared by the method of Bertolacini and Barney (1957).

All solvents were reagent grade and used without further purification except as noted below. Reagent grade acetic anhydride (Fisher, St. Louis) was distilled before use. Reagent grade hexane (Fisher) was distilled from potassium permanganate and the fraction boiling between 68.5 and 70.0° collected. Acetone for isopropylidine synthesis was purified on an alumina column and distilled from potassium permanganate. All solvents were mixed in proportion by volume.

Silicic acid, 100 mesh (Mallinkrodt, St. Louis), was dried overnight at 100° and stored in tightly closed containers. Florisil (Floridin Co., Tallahassee, Fla.) was washed as described by Wells and Dittmer (1965a). Thin layer chromatography was carried out on 0.5-mm thick, silica gel G (E. Merck, A. G., Darmstadt, Germany) plates. Bio-Rad AG 1-X2, 100–200 or 200–400 mesh, ion-exchange resin (Calbiochem, Los Angeles) was washed in succession with 6 N HCl (2 l./500 g), water until the effluent was acid free, 3 M sodium formate until the effluent was chloride free, and water (5 l./500 g). Bio-Rad AG 50W-X4 exchange resin was washed in succession with 6 N HCl (10 ml/g), water until neutral, 6 N NaOH (10 ml/g), and water until neutral.

Cerebroside, sulfatide, and sphingomyelin were prepared by the method of Wells and Dittmer (1965a). Ceramide was prepared by hydrolysis of sphingomyelin with *Clostridium perfringens* type A toxin (Lederle Laboratories, Pearl River, N. Y.) as described by Sribney and Kennedy (1958) and purified by chromatography. The ether-soluble enzyme reaction products were dissolved in chloroform and applied to a silicic acid column (Hanahan *et al.*, 1957). After washing the column with chloroform, the ceramide was eluted with $CHCl_3$–CH_3OH (19:1). The product had 2.19% nitrogen (lignocerylsphingosine has 2.16% nitrogen) and less than 0.1% phosphorus. Bovine erythrocyte "phosphatidylethanolamine" was prepared by the method of Hanahan *et al.* (1960). Rat brain phosphatidylcholine was prepared from a chloroform–methanol extract by chromatographing a sample of the extract containing 280 μmoles of phosphorus on a 10-g, 1.2 × 20 cm alumina column followed by chromatography on silicic acid. The lipid was placed on the alumina column in chloroform–methanol (3:2) and the column eluted with 400 ml of the same solvent. The effluent which also contained sphingomyelin and neutral lipids was concentrated and rechromatographed on a 5-g silicic acid column (Hanahan *et al.*, 1957). The phosphatidylcholine was eluted from the column with 180 ml of chloroform–methanol (3:2) after eluting the neutral lipids with 75 ml of chloroform. The sphingomyelin was retained on the column. Barium salts of acidic phospholipids were prepared from a diethyl ether–methanol-soluble fraction of a chloroform–methanol extract of sheep brain (Wells and Dittmer, 1965b) as described by Macfarlane (1961). Galactosyldiglyceride and 1,2-palmitalglycerol were gifts from Dr. M. Kates and Dr. D. N. Rhodes, respectively. Glycerolphosphorylglycerol was made available by Dr. R. L. Lester who obtained it from Dr. A. A. Benson.

Analytical Methods. Phosphorus was determined by the method of Fiske and Subbarow (1925) after digestion with perchloric acid. Fractions from AG-1 exchange-resin columns were analyzed by the method of Bartlett (1959) after digesting the dried samples by refluxing in 0.4 ml of 70–72% perchloric acid for 20 min. Samples with a low phosphorus content or containing mercuric chloride were treated as described by Dawson *et al.* (1962). Hexose was assayed by the method of Radin *et al.* (1956) as modified by Wells and Dittmer (1963). Although this method is satisfactory for purified samples of glycolipid, the assay mixture was often cloudy when total brain lipids were assayed. This could be eliminated by extracting the acid hydrolysate twice with 0.5 ml of diethyl ether. Nitrogen was assayed by direct nesslerization (Lang, 1958) as modified by Wells and Dittmer (1965a). Glucosamine was determined by the Elson–Morgan reaction (Svennerholm, 1956) and *N*-acetylneuraminic acid with resorcinol reagent as described by Svennerholm (1957). In the latter assay the chromogen was extracted into butyl ether–butanol (Miettiner and Takki-Luukkainen, 1959). Glycerol and inositol were assayed as described elsewhere (Wells and Dittmer, 1965b). Cholesterol and cholesterol ester were assayed by the Liebermann–Burchard reaction coupled with digitonide precipitation (Sperry and Webb, 1950). In the cholesterol ester assay the cholesterol was separated from the neutralized solution (Sperry and Brand, 1943) as recommended by Rodnight (1957).

When cholesterol was assayed in total lipid extracts, the phospholipids were removed by the method of Johnson *et al.* (1948). This step was not necessary when the chloroform effluent from Florisil columns was assayed. Fatty alkyl glyceryl ethers were assayed by gas–liquid partition chromatography of isopropylidine derivatives (Hanahan *et al.*, 1963). Free glyceryl ethers were obtained by acetolysis–saponification (Thompson and Lee, 1965).

Plasmalogens were assayed by several methods. The vinyl ether linkage was assayed by iodine uptake as described by Rapport and Franzl (1957) and Gottfried and Rapport (1962). The two methods agreed within 5%. Aldehyde was assayed by the method of Korey and Wittenburg (1954) as modified by Rapport and Alonzo (1955) using the *p*-nitrophenylhydrazone of palmitaldehyde as a standard. When greater sensitivity was required, the Schiff's assay of Marinetti *et al.* (1957) as modified by McIlwain and Rodnight (1962) was used with 1,2-palmitalglycerol as a standard. In the application of this method for the determination of the cyclic acetal of glycerophosphorylethanolamine, sulfatide, which is also present, did not interfere. Total fatty acids and acyl esters were determined by methods previously described (Wells and Dittmer, 1963).

Sulfate was assayed by a modification of the method of Spencer (1960). Samples containing 0.1–0.5 μmole of sulfate were hydrolyzed and extracted by the method of Long and Staples (1961). The aqueous phase was taken to dryness in a steam bath with the aid of a jet of air. To ensure the removal of HCl, the residue was dissolved in 0.5 ml of methanol–water (1:1) and dried again. This step was repeated two more times. It is imperative that all the HCl be removed. The residue was then dissolved in 0.1 ml of water, and 0.4 ml of ethanol and 0.05 ml of 0.5 N acetate buffer, pH 4.0, containing 0.5 μmole of K_2SO_4/ml were added in succession. To this was then added 0.05 ml of 0.5 N acetate buffer, pH 4.0, containing 20 mg/ml of barium chloranilate. After standing with occasional shaking at room temperature for 20 min, the suspension was centrifuged at 300*g* for 10 min. The supernatant solution (0.2 ml) was removed and diluted with 2.0 ml of 0.5 N acetate buffer, pH 4.0, and the absorption was measured at 327.5 mμ. This method could not be used unless the phospholipids were removed with Florisil first. Apparently the phosphate produced by acid hydrolysis of phospholipids exceeds the buffering capacity of the reaction mixture and chloranilate ion was released into solution. The buffering capacity of the assay mixture could not be increased because the barium chloranilate was more soluble in solutions of higher ionic strength and as a result the blank became too high.

Periodate was determined by the arsenite–iodine method of Fleury and Lange (1933). All solutions containing periodate were prepared just before use and stored in containers wrapped in aluminum foil. All manipulations of these solutions were carried out in a darkened room. An Ultra-Buret (Scientific Industries, Springfield, Mass.) was used for the titrations. L-3-Glycerol phosphate was determined with rabbit muscle glycerol phosphate dehydrogenase as described by Wichert (1962). Glycerol phosphate eluted from anion-exchange columns with buffers containing borate could not be assayed directly because borate interferred. Borate was removed with anion-exchange resin (Hawthorne and Hübscher, 1959) and its absence demonstrated as described by Hübscher and Hawthorne (1957). Glycerol bonded with the primary hydroxyl group only was determined by formaldehyde production after periodate oxidation. The sample in 1.0 ml of 0.1 M sodium acetate buffer, pH 4.7, was mixed with 0.25 ml of 0.1 M sodium periodate. After standing at room temperature for 30 min, 0.25 ml of 10% sodium bisulfite was added and duplicate 0.5-ml samples were treated with 2.5 ml of chromotropic acid reagent (Frisell *et al.*, 1954) for the determination of formaldehyde.

Chromatographic Methods. Carbohydrates were separated and identified by chromatography on Whatman No. 1 paper with the ethyl acetate–pyridine–water system of Jermyn and Isherwood (1949). Free carbohydrates were obtained by hydrolyzing in 3 N H_2SO_4 at 100° for 2 hr. The sulfate was removed from the hydrolysate with AG-3 anion-exchange resin. For the detection of monogalactosylglycerol, mild alkaline hydrolysates were freed of sodium with AG-50 (Dawson, 1960) and chromatographed in ethyl acetate–pyridine–water or the isopropyl alcohol–acetic acid–water system of Kaufman *et al.* (1965). The alkaline silver nitrate reagent of Trevelyan *et al.* (1950) was used for the detection of carbohydrates.

High-voltage paper electrophoresis was carried out as described by Dawson and Dittmer (1961). Samples were prepared by hydrolyzing with 6 N HCl for 2 hr at 100° and then removing the excess HCl with an air jet. Phosphorus-containing compounds were detected with the perchloric acid–ammonium molybdate spray of Hanes and Isherwood (1949), amino compounds with 0.2% ninhydrin in acetone, and choline and compounds-containing choline with the phosphomolybdate reagent of Chargaff *et al.* (1948).

Several solvent systems were used for silica gel thin layer plates. Neutral lipids were separated with ethylene dichloride (tank saturation) or ethylene dichloride–methanol (98:2) (Jatzkewitz and Mehl, 1960), ceramide was chromatographed with chloroform–acetone (2:1) (Fujino and Zabin, 1962), and polar lipids were separated with either chloroform–methanol–7 N NH_4OH (65:35:5) (Skidmore and Entenman, 1962) or chloroform–methanol–H_2O–pyridine (40:10:1:1) (Dittmer and Lester, 1964). Lipids were detected as described by Dittmer and Lester (1964).

Extraction of Lipids. Unanesthetized, male Sprague-Dawley rats were decapitated and the brains removed and frozen in Dry Ice within 30 sec. The frozen brains were weighed and extraction was begun within 10 min. The chloroform–methanol, chloroform–methanol–HCl extraction procedure described by Wells and Dittmer (1965b) was used. As pointed out by us in describing

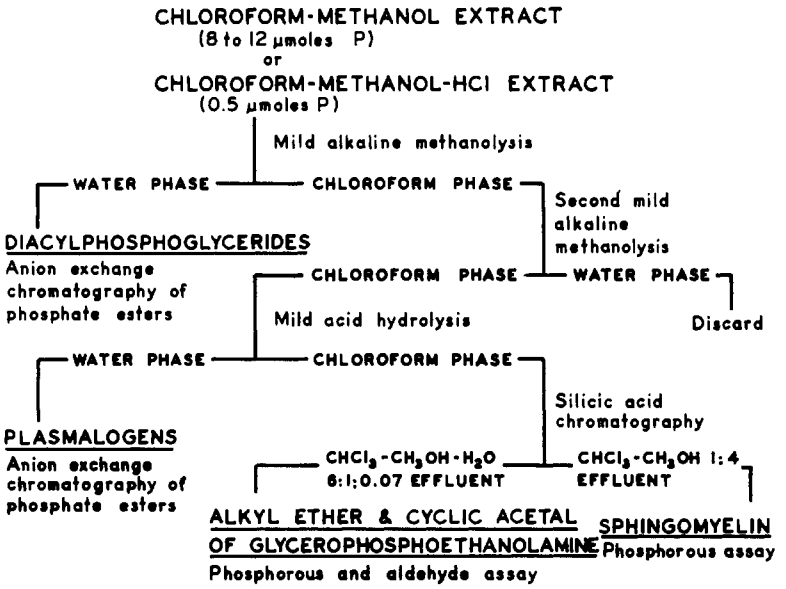

FIGURE 1: Diagram of analysis procedure for brain phospholipids.

this extraction procedure, the quantitative analysis of the polyphosphoinositides requires that the brain be handled in a manner to give minimum autolysis. Once extracted, the extracts could be stored at −18° for several weeks with no detectable change in lipid composition. In the case of large-scale sheep brain lipid preparations, the brains were obtained within 10 min after slaughter and extraction was begun within 30 min by the same procedure except that nonlipid contaminants were removed from the chloroform–methanol extracts by partitioning with water (Folch *et al.*, 1957). These preparations were taken to dryness *in vacuo* at room temperature and suspended in chloroform–methanol (2:1), and the insoluble material removed by filtering on a sintered glass filter. Nonlipid contaminants were removed from small preparations with Sephadex (Wells and Dittmer, 1963). Generally these lipid extracts were not taken to dryness after treatment with Sephadex but simply made to a known volume with chloroform–methanol (2:1).

Microanalysis of Brain Lipids. A. ANALYSIS OF PHOSPHOLIPIDS. Individual phospholipids were analyzed by a selective hydrolysis procedure similar to that used by Dawson (1960) coupled with the fractionation of the water-soluble phosphate esters on anion-exchange resin as described by Lester (1963) and silicic acid chromatography of the stable lipids. The general outline of the scheme used is given in Figure 1.

The mild alkaline methanolysis of the diacyl lipids was based on the method of Brockerhoff (1963). A sample of the chloroform–methanol extract with 8–12 μmoles of phosphorus or of the chloroform–methanol–HCl extract with 0.5 μmole of phosphorus was taken to dryness and suspended in 1 ml of chloroform–methanol (1:4). Then 0.1 ml of 1.2 N NaOH in methanol–water (1:1) was added and the mixture incubated at 37° for 10 min. The mixture was neutralized by the addition of 0.15 ml of 1 N acetic acid and 2 ml of chloroform–methanol (9:1), 1 ml of isobutyl alcohol, and 2 ml of water were added. The mixture was shaken and then centrifuged for 10 min at 300*g*. The upper phase was drawn off with a Pasteur pipet and the lower phase was reextracted two times with 1 ml of methanol–water (1:2). The upper aqueous phases were combined and taken to dryness *in vacuo* after adding 0.5 ml of isoamyl alcohol to prevent foaming. The residue was dissolved in 2 ml of 0.02 M ammonium borate, pH 9.5, for fractionation of the phosphate esters.

The lower or chloroform phase from the mild alkaline methanolysis of the chloroform–methanol–HCl extract was discarded. This phase from the chloroform–methanol extract was taken to dryness with the aid of a jet of nitrogen and subjected to a second mild alkaline hydrolysis to assure complete deacylation of the plasmalogens and the alkyl ether analog of phosphatidylethanolamine. The procedure was the same as that described above except the incubation was carried out for 15 min. The upper phases were discarded, the lower phase taken to dryness and redissolved in 1.6 ml of chloroform–methanol (5:11), and 0.4 ml of 0.025 M $HgCl_2$ in 0.05 N HCl was added. The mixture was incubated at 37° for 15 min and then 1.5 ml of chloroform and 1.6 ml of water were added. This mixture was shaken vigorously and then centrifuged and the upper phase was drawn off. The lower phase was reextracted with 1 ml of methanol–water (1:1). The upper phases were combined, 0.1 ml of 0.1 M sodium ethylenediaminetetraacetic acid, pH 7, 0.05 ml of ammonium hydroxide, and 0.5 ml of isoamy lalcohol were added, and the mixture was taken dryness *in vacuo*. The residue was dissolved in 2.0 ml

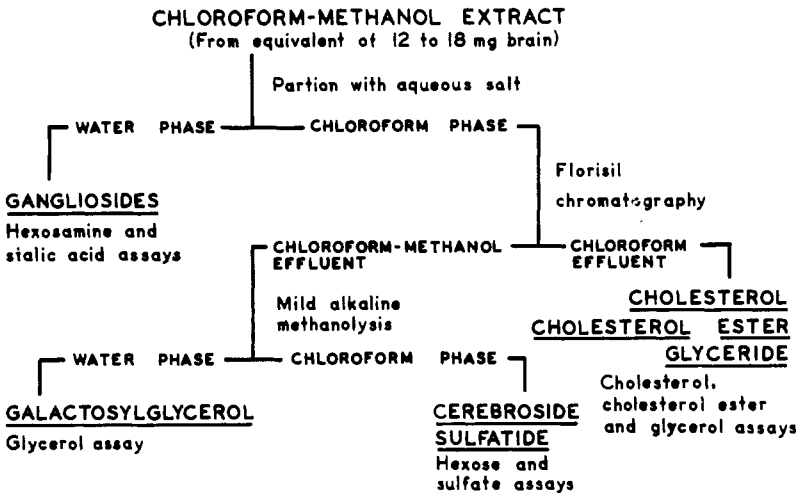

FIGURE 2: Diagram of analysis procedure for neutral brain lipids.

of 0.02 M ammonium borate, pH 9.5, for fractionation by anion-exchange chromatography. The addition of sodium ethylenediaminetetraacetic acid prevented the formation of a precipitate when the solution was neutralized with ammonium hydroxide.

The lower phase obtained after acid hydrolysis contained sphingomyelin, alkyl ether, and cyclic acetalglycerophosphorylethanolamine. These compounds were separated by silicic acid chromatography. The solution was taken to dryness under nitrogen, and the residue was dissolved in chloroform and placed on a column of 2 g of silicic acid and 1 g of Hyflo Super Cel (Hanahan *et al.*, 1957). The column was eluted with 20 ml of chloroform, 50 ml of chloroform–methanol–water (11:1:0.06), 60 ml of chloroform–methanol–water (6:1:0.07), and 30 ml of chloroform methanol (1:4). Each solvent was collected in bulk at a flow rate of 0.5 ml/min. The first two fractions contain no detectable phosphorus and were discarded. The last two fractions were made to 5 and 10 ml, respectively, and 1-ml samples taken in duplicate from each fraction for phosphorus assay. Additional 1-ml samples were taken in duplicate from the chloroform–methanol–water (6:1:0.07) fraction for aldehyde assay (Marinetti *et al.*, 1957).

Ion-exchange chromatography of the phosphate esters obtained after mild alkaline hydrolysis and mild acid hydrolysis of the chloroform–methanol extract and of the chloroform–methanol–HCl extract was carried out by the method of Lester (1963). We are indebted to Dr. Lester for details of this procedure not yet published. The water-soluble products obtained after mild alkaline and acid hydrolysis of the chloroform–methanol extract were separated on a 0.4 × 80 cm column of Bio-Rad AG 1-X2, 200–400 mesh, formate anion-exchange resin. The columns were packed and the flow rate was adjusted to 0.4 ml/min using 0.1 M ammonium formate–0.02 M borate adjusted to pH 9.5 with ammonium hydroxide. The sample in 2 ml of 0.02 M borate was washed into the column with two 0.5-ml samples of the same ammonium formate buffer. The columns were then eluted with a gradient of ammonium formate at pH 8.5. The gradient was mixed with three chambers of a Buchler Varigrad. In the first two chambers, 75 ml of 0.1 M ammonium formate–0.02 M borate, pH 8.5, was used, and 75 ml of 0.625 M ammonium formate–0.02 M borate, pH 8.5, buffer was used in the third chamber. The entire gradient was collected in 2-ml fractions. The water-soluble mild alkaline methanolysis products of the acidified chloroform–methanol extract were separated as described above except that a different gradient was used. The columns were packed and the flow rates adjusted with 0.12 M ammonium formate–0.02 M borate, pH 9.5. The samples were washed on with the same buffer. In this case 75 ml of this buffer was used in the first two chambers and 0.675 M ammonium formate–0.02 M borate, pH 9.5, buffer was used in the third chamber. The entire gradient was collected in 2-ml fractions. Preparative columns were run using 0.8 × 80 cm columns and 150 ml of sodium formate instead of ammonium formate buffers in each chamber.

B. ANALYSIS OF CHOLESTEROL, CHOLESTEROL ESTER, GANGLIOSIDE, GLYCERIDE, CEREBROSIDE, SULFATE, AND GALACTOSYLGLYCERIDE. For the determination of all the lipids other than phospholipids another sample of the chloroform–methanol extract which contained 8–12 μmoles of phosphorus was used (Figure 2). This sample was the equivalent of the extract from 12 to 18 mg wet weight of whole adult rat brain. The gangliosides were removed from this sample by essentially the procedure described by Folch *et al.* (1957). The lipid in 3 ml of chloroform–methanol–water (60:30:4.5) was mixed thoroughly on a Vortex mixer with 0.5 ml of 0.1 N KCl. The emulsion was separated by centrifuging at 300*g* for 10 min and the upper, water phase was withdrawn with a Pasteur pipet. The lower phase was partitioned three times more with 1 ml of chloro-

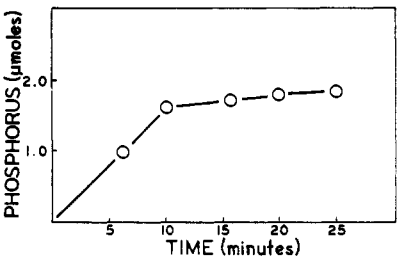

FIGURE 3: The effect of the duration of mild alkaline methanolysis on the subsequent release of phosphorus by mild acid hydrolysis of the chloroform-soluble products. Each point represents a sample of a chloroform–methanol extract of rat brain containing 10 μmoles of phosphorus which was treated under mild alkaline conditions for the indicated times and the chloroform-soluble fraction then subjected to mild acid hydrolysis for 15 min and partitioned. The hydrolysis and extraction conditions are given in the text.

form–methanol–0.1 N KCl (1:10:10) and the combined water phases were made to a final volume of 5 ml. Duplicate 1-ml samples were taken for analysis of hexosamine and neuraminic acid as a measure of ganglioside.

The chloroform phase from the ganglioside extraction was separated into neutral lipids and glycolipids on Florisil (Radin *et al.*, 1956; Rouser *et al.*, 1961). Specifically the chloroform phase was taken to dryness at room temperature with the aid of a jet of nitrogen and the residue, dissolved in chloroform, was quantitatively transferred to a 1-g Florisil column (0.7 × 5.0 cm) which had been made up in chloroform. The column was eluted with 20 ml of chloroform followed by 20 ml of chloroform–methanol (2:1). Both fractions were collected in bulk at a flow rate of 0.5 ml/min and taken to dryness *in vacuo* at room temperature. The chloroform fraction was made to a final volume of 5 ml and duplicate 1-ml samples were taken for the analysis of cholesterol–cholesterol ester and glycerol. The glycerol was used as a measure of glyceride content.

The residue of the chloroform–methanol (2:1) fraction from the Florisil column was dissolved in 3 ml of chloroform–methanol (2:1) and 0.3 ml of 1.2 N NaOH in methanol–water (1:1) was added. The mixture was incubated for 15 min at 37° and then neutralized with 0.3 ml of 1.2 N acetic acid. Isobutyl alcohol (1 ml) and 2 ml of water were then added and the mixture was shaken and centrifuged for 10 min at 300*g*. The upper aqueous phase was drawn off and the lower phase was reextracted with 1 ml of methanol–water (1:1). The combined water phases were made to a final volume of 5 ml with water and the duplicate 2-ml samples were assayed for glycerol as a measure of the galactosylglyceride. The lower chloroform phase was made to 5 ml and duplicate 1-ml samples were taken for the assay of sulfate and galactose. The sulfate was used as a measure of the cerebroside sulfate and the galactose minus the sulfate as a measure of cerebroside.

Results

Evaluation of Phospholipid Analysis. The quantitative analysis of phospholipids based on the analysis of water-soluble phosphate esters which are characteristic of the parent phospholipid has been used extensively by Dawson (1960) and Dawson *et al.* (1962). In this method selective mild alkaline hydrolysis of the fatty acyl esters gave rise to water-soluble phosphate esters from the diacyl phosphoglycerides, while the plasmalogens and alkyl ether phospholipids gave rise to chloroform-soluble lyso compounds and sphingomyelin was unchanged. Subsequent mild acid hydrolysis of the chloroform-soluble products gave rise to water-soluble phosphate esters from the lysoplasmalogens and chloroform-soluble, unchanged lysoalkyl ether phospholipids and sphingomyelin. Dawson separated the phosphate esters from the diacyl phosphoglycerides and plasmalogens on paper for quantitative analysis. The chloroform-soluble products after the acid hydrolysis were analyzed by taking advantage of the fact that after further strong acid hydrolysis the sphingomyelin gave rise to water-soluble phosphorus compounds while the alkyl ether phospholipid phosphorus remained chloroform soluble.

Several important criteria must be met before this method or any modification can be successfully applied to the quantitative analysis of phospholipids. First, the alkaline hydrolysis must be selective with only a minimum of hydrolysis of the phosphate esters and must quantitatively cleave the acyl esters of the plasmalogens and alkyl ether phospholipids, as well as those of the diacyl phosphoglycerides. Second, the mild acid hydrolysis must also be selective and must quantitatively cleave the vinyl ether linkage with a minimum of cyclic acetal formation. Third, each solvent partition must quantitatively separate chloroform and water-soluble products. Fourth, the water-soluble phosphate esters produced by each hydrolysis step must be separated and quantitatively determined. Fifth, the sphingomyelin and lysoalkyl ether phospholipids must be separated and quantitatively determined. All of these criteria were checked.

A. EVALUATION OF THE PROCEDURE FOR SELECTIVE HYDROLYSIS OF PHOSPHOLIPIDS. Ukita *et al.* (1955) and Mauro and Benson (1959) showed that prolonged hydrolysis in methanolic sodium hydroxide caused breakdown of glycerophosphorylcholine to glycerophosphorylmethanol and cyclic glycerophosphate. Brockerhoff (1963) showed that the deacylation of phosphoglycerides was best carried out in a solvent of high methanol content, low base concentration, and as short a reaction time as was commensurate with complete methanolysis. The release of water-soluble phosphorus from diacyl phosphoglycerides during mild alkaline methanolysis using the conditions finally adopted here showed that methanolysis was complete in 10 min. Under these conditions less than 1% break-

down of glycerophosphorylcholine occurred as determined by hydrolysis of purified phosphatidylcholine. Dawson (1960) reported significant breakdown of glycerophosphorylinositol and Dawson and Dittmer (1961) observed breakdown of glycerophosphorylinositol diphosphate using Dawson's procedure. Examination of hydrolysates of purified phosphatidylinositol and triphosphoinositide by column chromatography and paper electrophoresis failed to show any breakdown of these compounds with the methanolysis conditions used here.

Although 10 min was sufficient for complete methanolysis of the ester linkages of the diacyl phosphoglycerides, the ester linkage of the plasmalogens was more resistant. This has been noted by Ansell and Spanner (1963b) and Renkonen (1963). The alkyl ether phospholipids show this same property (Hanahan *et al.*, 1963). Figure 3 shows the effect of varying the time of alkaline methanolysis on the release of water-soluble phosphorus during subsequent mild acid hydrolysis. It was evident that at least 25 min was required to completely hydrolyze the ester linkages of the plasmalogens. Because this longer period of alkaline methanolysis led to considerable breakdown of glycerophosphorylcholine, two separate alkaline methanolyses were used. In this method a 10-min methanolysis was used to release the phosphate esters from the diacyl phosphoglycerides. After the water-soluble products were removed, the chloroform-soluble products were subjected to a second alkaline methanolysis for 15 min. This completely hydrolyzed the ester linkage of the plasmalogens as established in the following way. Plasmalogens not deacylated are still soluble in chloroform after mild acid hydrolysis. Alkaline hydrolysis of the chloroform-soluble product produced by acid hydrolysis would deacylate these products giving rise to water-soluble phosphorus. When this was done no phosphorus could be detected in the water phase.

The release of water-soluble phosphorus during mild acid hydrolysis of the lysoplasmalogens showed that hydrolysis was complete after 15 min. The yield of water-soluble phosphorus was 103% of that calculated from the aldehyde and vinyl ether content of the starting material.

In addition to the water-soluble compounds, the plasmalogens can also give rise to cyclic acetals during acid hydrolysis (Davenport and Dawson, 1962; Pietruszko and Gray, 1962). Davenport and Dawson reported that the inclusion of mercuric chloride in the acid hydrolysis mixture reduced the formation of cyclic acetal. This was confirmed by isolation of the cyclic acetal by silicic acid chromatography. In the presence of mercuric chloride no more than 1% of ethanolamine plasmalogen was accounted for as the cyclic acetal. In the absence of mercuric chloride up to 40% cyclization occurred.

In attempting to obtain data on the quantitative hydrolysis of lysoplasmalogens, it was noted that the yield of water-soluble phosphorus was lower after acid hydrolysis than could be accounted for on the basis of incomplete ester hydrolysis or cyclic acetal

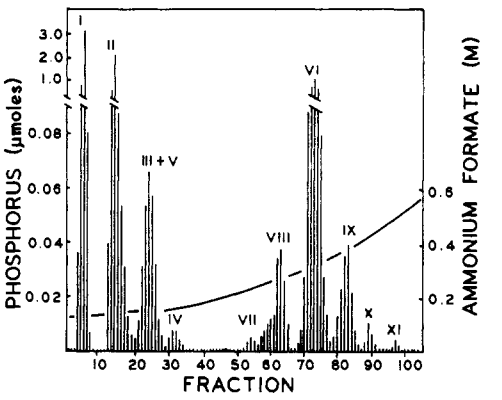

FIGURE 4: Anion-exchange fractionation at pH 9.5 of the water-soluble, mild alkaline methanolysis products of a chloroform–methanol extract of rat brain by the method of Lester (1963). A sample of the extract containing 10 μmoles of phosphorus was hydrolyzed and of this 7 μmoles was soluble in the fraction chromatographed. The individual peaks are identified in Table I.

formation. All these experiments were carried out using chloroform–methanol–water mixtures to separate the mild alkaline hydrolysis products. When the recovery of aldehyde in the chloroform phase after mild alkaline hydrolysis was checked, it was found that about 10% had been lost. Because no loss of aldehyde or vinyl ether was detected if the mild alkaline hydrolysate was neutralized and analyzed without separation of the water- and chloroform-soluble products, it was concluded that this loss was due to extraction of the lysoplasmalogen into the water phase. A larger loss was observed if the alkaline hydrolysate was extracted without first neutralizing. The alkyl ether analog of lysophosphatidic acid is also extracted into basic aqueous solutions (D. J. Hanahan, personal communication, 1964). The loss encountered when neutralized mild alkaline hydrolysates were extracted could be prevented by adding isobutyl alcohol to the mixture before extraction, carrying out subsequent extractions with methanol–water (1:2). Dawson (1960) used isobutyl alcohol in a similar application in his procedure; however, for some unaccountable reason lysoplasmalogen was lost when his procedure was checked. This solvent change did not prevent losses if the mild alkaline hydrolysate was not neutralized. It is therefore important to neutralize to a pH between 6.5 and 7.0 before extraction. When this is done and the change in solvent is made, less than 0.5% loss is encountered.

B. SEPARATION AND IDENTIFICATION OF THE WATER-SOLUBLE MILD ALKALINE METHANOLYSIS PRODUCTS OF THE CHLOROFORM–METHANOL EXTRACT. Dawson (1960) separated water-soluble phosphate esters by a combination of paper chromatography and electrophoresis. The compounds were then detected on the paper and cut out, and their phosphorus content was determined. This method has two disadvantages. First, it is of little

TABLE I: Chemical Characterization of AG-1 Fractions from Mild Alkaline Methanolysates of the Chloroform–Methanol Extract.

Fraction	Compound	Molar Ratios Found[a]						Nitrogen Component
		pH 8.5 Column			pH 9.5 Column			
		N	Inositol	P	N	Inositol	P	
I	Glycerophosphorylcholine	1.03	—	1.01	1.06	—	1.00	Choline
II	Glycerophosphorylethanolamine	—	—	—	1.01	—	1.02	Ethanolamine
III	Glycerophosphorylinositol	—	1.04[b]	0.99				
IV	Glycerophosphorylglycerol	—	—	0.51		Not resolved		
V	Artifact	—	—	1.01				
VI	Glycerophosphorylserine	1.03	—	0.98	0.97	—	0.99	Serine
VII	Inorganic phosphate	—	—	—	—	—	—	
VIII	Glycerol phosphate	—	—	1.02	—	—	1.04	
IX	Bis(glycerophosphoryl)-glycerol	—	—	0.67	—	—	0.69	
X	Glycerophosphorylglycerol phosphate	—	—	0.99	—	—	—	
XI	Glycerophosphorylinositol phosphate	—	—	—	—	1.11[c]	1.96	

[a] Glycerol has been assayed on all the samples and assigned a value of 1.00. All other ratios are in reference to glycerol. [b] Determined as trimethylsilyl derivative. [c] Determined microbiologically.

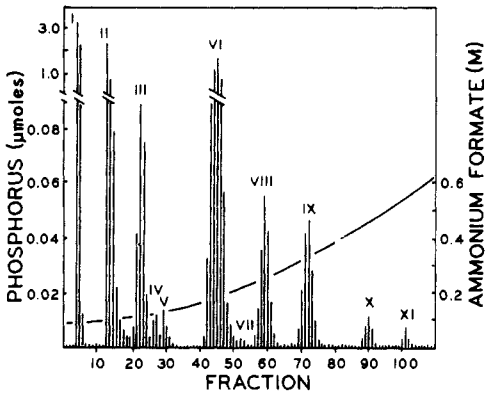

FIGURE 5: Anion-exchange fractionation at pH 8.5 of the water-soluble mild alkaline methanolysis products of a chloroform–methanol extract of rat brain. The sample chromatographed is described in Figure 4. The individual peaks are identified in Table I.

value for the determination of minor components since the capacity of the paper and sensitivity of the detecting reagent is low. This is borne out by Dawson's failure to detect glycerophosphorylglycerol, glycerophosphorylglycerol phosphate, and glycerolphosphorylinositol phosphate in hydrolysates of chloroform–methanol extracts of brain. Second, some of the compounds are not well separated.

The use of anion-exchange chromatography to separate phosphate esters derived from phospholipids by mild alkaline hydrolysis was introduced by Hübscher and Hawthorne (1957). Several different methods have appeared subsequently (Hawthorne and Hübscher, 1959; Hübscher *et al.*, 1960; Brockerhoff and Ballou, 1961; Ellis *et al.*, 1963; Lester, 1963; Chang and Sweeley, 1963). Of these, the method of Lester was used because it employs a simple, highly reproducible gradient and the separation achieved with yeast phospholipids was excellent. The method of Lester uses a three-chamber gradient in which 0.12 M ammonium formate–0.02 M borate buffer, pH 9.5, was used in the first two chambers and 0.675 M ammonium formate buffer, pH 9.5, in the third. The separation of the water-soluble phosphate esters derived from a chloroform–methanol extract of brain lipids is shown in Figure 4. There are two points which limit the usefulness of this particular gradient. A hydrolysis artifact derived from glycerophosphorylcholine was not separated from glycerophosphorylinositol, and a clean separation of (bis-glycerophosphoryl)glycerol and glycerophosphorylserine was not always achieved. This was probably because the concentration of phosphatidylserine is much higher in brain than in yeast. At the suggestion of Dr. Lester, a gradient using a pH 8.5 buffer was tried; this gave improved separation. If the gradient were made with 0.10 M ammonium formate–0.02 M borate buffer, pH 8.5, in chambers one and two and 0.625 M ammonium formate–0.02 M borate, buffer pH 8.5, in chamber three, the separation shown in Figure 5 was obtained. In this case, all the components were well separated except glycerophosphorylinositol.

TABLE II: Chemical Characterization of AG-1 Fractions from Mild Acid Hydrolysates of the Chloroform–Methanol Extract.

Fraction	Compound	Molar Ratios Found				Nitrogen Component
		N	Inositol	P	Glycerol	
I	Glycerophosphorylcholine	—	—	1.03	1.00	Choline
II	Glycerophosphorylethanolamine	1.03	—	1.01	1.00	Ethanolamine
III	Glycerophosphorylinositol	—	1.02[a]	0.98	1.00	
VIII	Glycerol phosphate	—	—	1.02	1.00	

[a] Determined as trimethylsilyl derivative.

However, this was not serious enough that these components could not be analyzed, and this gradient was chosen as a compromise. Table I gives the analyses of the fractions isolated by preparative runs on the water-soluble mild alkaline hydrolysis products of the chloroform–methanol extract of brain. While the initial identity of each peak was made on the basis of these analyses, additional information on the identity of peaks IV, V, IX, and X was obtained because they either had not been reported previously in brain or where reported had not been adequately characterized.

Phosphatidylglycerol was first reported by Mauro and Benson (1957) and Benson and Mauro (1958). It has been reported in animal tissue by Strickland and Benson (1960) and Gray (1964) but it has not been reported previously in brain. A compound was detected among the water-soluble, mild alkaline methanolysis products of brain lipids, peak IV, Figure 5, which cochromatographed with authentic glycerophosphorylglycerol at pH 8.5 and 9.5. Since this compound was not well separated from peaks III and V, isolation of pure material from a total brain lipid hydrolysate was not possible. A pure sample of glycerophosphorylglycerol was obtained from the water-soluble mild alkaline methanolysis products of a barium salt fraction of sheep brain lipids by preparative AG-1 chromatography. The isolated material had a molar phosphorus–glycerol ratio of 0.51, and periodate oxidation yielded 1.97 μmoles of formaldehyde/μmole of phosphorus. All attempts to obtain the intact lipid in order to determine fatty acid content were unsuccessful; however, when the acidic lipids of sheep brain obtained by barium salt fractionation were chromatographed on silicic acid and the water-soluble deacylation products of each fraction chromatographed on anion-exchange resin, the fraction yielding glycerophosphorylglycerol was found to have been eluted with the same solvent which elutes *Escherichia coli* phosphatidylglycerol.

Peak V, Figure 5, is an artifact of mild alkaline methanolysis which is derived mostly from glycerophosphorylcholine. Using methanolysis conditions in which the solvent contained a high proportion of chloroform or a higher concentration of sodium hydroxide this peak was increased. This was true whether purified phosphatidylcholine or the total chloroform–methanol extract was hydrolyzed. The increase in this peak accounted for 99% of the phosphorus lost from the glycerophosphorylcholine peak when purified phosphatidylcholine was hydrolyzed. The remaining 1% was glycerol phosphate. When chloroform–methanol extracts were hydrolyzed, 90% of the phosphorus in this peak accounted for the loss from the glycerophosphorylcholine peak and the rest was derived from glycerophosphorylethanolamine. With the methanolysis conditions finally adopted, there was 0.5–1% breakdown of glycerophosphorylcholine. Since only 1% of the breakdown gave rise to glycerol phosphate, the amount of glycerol phosphate which would erroneously be calculated as phosphatidic acid was less than 1% of the glycerol phosphate found.

Diphosphatidylglycerol. Dawson (1960) found a compound in the water-soluble deacylation products of brain lipids which chromatographed like bis(glycerophosphoryl)glycerol. No further characterization or evidence for the occurrence of diphosphatidylglycerol in brain is available. Bis(glycerophosphoryl)glycerol, peak IX, Figure 5, was isolated and characterized. It had a molar phosphorus/glycerol ratio of 0.67. The compound consumed 1.01 μmoles of sodium periodate/μmole of phosphorus in phosphate buffer, pH 6.6, and 0.99 μmole of formaldehyde was produced/μmole of periodate consumed. No hydrolysis of the compound with alkaline phosphatase could be detected. Periodate oxidation and dimethylhydrazinolysis (Le Cocq and Ballou, 1964) produced glycerol diphosphate which was isolated in 72% yield by AG-1 chromatography. It had a molar phosphorus/glycerol ratio of 2.02 and no detectable formaldehyde was produced by periodate oxidation. These data established that the bis(glycerophosphoryl)glycerol of brain had the same structure as that isolated from heart muscle cardiolipin (Faure and Morelec-Coulon, 1956; Morelec-Coulon and Faure, 1958; MacFarlane and Gray, 1957; Gray and MacFarlane, 1958; MacFarlane, 1958; MacFarlane and Wheeldon, 1959; Morelec-Coulon *et al.*, 1960; Le Cocq and Ballou, 1964).

Phosphatidylglycerol Phosphate. The identifica-

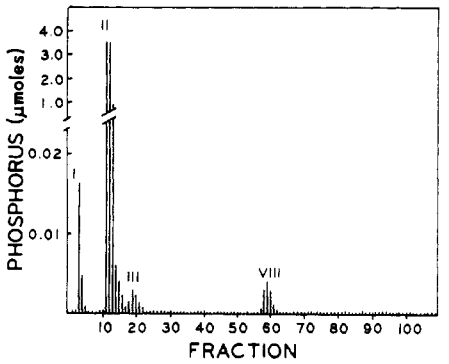

FIGURE 6: Anion-exchange chromatography at pH 8.5 of the water-soluble mild acid hydrolysis products of a chloroform–methanol extract of rat brain. A sample of the extract containing 10 μmoles of phosphorus was hydrolyzed by the procedure described in the text. The fraction chromatographed contained 2.5 μmoles of phosphorus. The individual peaks are identified in Table II.

tion of peak X, Figure 5, as 1-(L-3-glycerophosphoryl)-L-glycerol 3-phosphate has been given in detail elsewhere (Wells and Dittmer, 1966).

C. IDENTIFICATION AND CHARACTERIZATION OF THE MILD ACID HYDROLYSIS PRODUCTS OF THE CHLOROFORM–METHANOL EXTRACT. The separation of water-soluble products of the mild acid hydrolysis of lysoplasmalogens is shown in Figure 6, and Table II gives the analyses on these fractions. The identification of each peak was made on the basis of these analyses and additional information was obtained on the occurrence of choline plasmalogen, inositol plasmalogen, plasmalogenic acid, and serine plasmalogen.

Choline Plasmalogen. Peak I, Figure 6, corresponded to glycerophosphorylcholine chromatographically and analysis supported this identification. This fraction represented about 2% of the total choline phosphoglyceride in rat brain. More direct evidence for the occurrence of choline plasmalogen was obtained by analysis of a phosphatidylcholine preparation isolated from rat brain lipids by chromatography on alumina and silicic acid. This material analyzed for 1.85 moles of aldehyde/100 moles of phosphorus and had a molar vinyl ether/aldehyde ratio of 0.99. Ion-exchange chromatography showed that glycerophosphorylcholine produced by acid hydrolysis was 96% of that calculated from the aldehyde content of the starting material. The presence of significant concentrations of the alkyl ether analog was ruled out when less than 0.1% of the phosphorus was still chloroform soluble after mild alkaline and acid hydrolysis.

Inositol Plasmalogen. Presumptive evidence for the occurrence of this compound in brain was presented by Ohno (1952) who reported the presence of inositol in a lysoplasmalogen preparation from brain lipids. Peak III, Figure 6, was eluted from anion-exchange resin in the same position as glycerophosphorylinositol, and analysis of the fraction supported this identification. Attempts to prepare a monophosphoinositide fraction from rat brain lipids in order to demonstrate the plasmalogen more conclusively were not successful. However, the acidic lipid fraction of sheep brain prepared by barium salt fractionation and in which the monophosphoinositide was concentrated was enriched with inositol plasmalogen as indicated by anion-exchange fractionation of the acid hydrolysis products. This fraction represented 6.2% of the phosphorus in the water-soluble, mild acid hydrolysate of the acidic lipid preparation and on analysis was found to have a inositol/phosphorus/glycerol molar ratio of 0.98/1.02/1.00.

Plasmalogenic Acid. Hübscher and Clark (1960) reported that a nearly pure preparation of phosphatidic acid from rat liver contained 15% plasmalogen. This was greater than could be accounted for by the nitrogenous contaminants. Peak IV, Figure 6, was eluted at the position of glycerol phosphate, and this suggested the possible occurrence of plasmalogenic acid in brain. That this glycerol phosphate was derived from glycerophosphorylethanolamine during acid hydrolysis was ruled out because the amount of glycerol phosphate remained constant after 15, 30, and 45 min of acid hydrolysis. This would not be expected if it were formed by acid degradation of glycerophosphorylethanolamine. Further fractionation of brain lipids by solvent, salt, and chromatographic methods showed that the glycerol phosphate produced by acid hydrolysis was concentrated with the phosphatidic acid. For example, in sheep brain lipids, the glycerol phosphate accounts for 1.1% of the water-soluble phosphorus produced by mild acid hydrolysis, while in the barium salt fraction of these lipids, it accounts for 6.5%. The glycerol phosphate isolated by preparative AG-1 chromatography had a molar phosphorus/glycerol ratio of 1.02 and it assayed enzymatically as 97% L-3-glycerol phosphate.

Serine Plasmalogen. Dawson (1960) and Dawson *et al.* (1962) have reported serine plasmalogen in sheep and ox brain lipids on the basis of glycerophosphorylserine observed in mild acid hydrolysates. Although a small and inconsistent peak was observed in the region where glycerophosphorylserine was eluted from chromatograms of mild acid hydrolysates, serine plasmalogen appears to be present in rat brains in extremely small concentrations or not at all. That we could have detected it, if present, was established by the fact that it was detected in sheep brain lipids. In addition to observing the presence of glycerophosphorylserine in mild acid hydrolysates of sheep brain lipids, evidence was also obtained for the occurrence of serine plasmalogen by analyzing a barium salt fraction of the acidic lipids. The glycerophosphorylserine content of the acid hydrolysates from this fraction was consistent with the aldehyde content. This fraction had a fivefold enrichment of serine plasmalogen as compared with the original extract.

D. CHARACTERIZATION OF THE MILD ALKALINE HYDROLYSIS PRODUCTS OF THE CHLOROFORM–METHANOL–

HCl EXTRACT. The only phospholipids extract.d with acidified solvents were part of the diphosphoinositide and all of the triphosphoinositide. Characterization of the fractions obtained by deacylation of the extract and the conditions necessary for quantitative assay have been given in detail elsewhere (Wells and Dittmer, 1965b).

E. SEPARATION AND IDENTIFICATION OF THE CHLOROFORM-SOLUBLE, MILD ACID HYDROLYSIS PRODUCTS. The use of strong acid hydrolysis to determine the phosphorus of sphingomyelin and phosphoethanolamine glyceryl ether (Dawson *et al.*, 1962) does not distinguish between sphingomyelin and cyclic acetals derived from plasmalogens. Because the formation of small amounts of cyclic acetals could not be avoided, a silicic acid column was used to separate these lipids. Three phosphorus-containing peaks were eluted (Figure 7). Peak III was sphingomyelin and well separated from two close running compounds eluted with chloroform–methanol (6:1), 1% water. Peak I of this fraction but not peak II contained aldehyde. Both peaks were grossly contaminated with cerebroside sulfate as revealed by thin layer chromatography. Pooled samples from each peak were separated from cerebroside sulfate on preparative thin layer silica gel plates developed with chloroform–methanol–7 N NH_4OH. The compounds were located on the plates with water, the silica gel was scraped from the plate, and the lipid eluted with chloroform–methanol (1:9). The purified material which corresponded to peak I had a molar aldehyde/glycerol/phosphorus ratio of 0.97/0.99/1.00. It contained ethanolamine as determined by paper electrophoresis of an acid hydrolysate, no detectable fatty acids as determined by gas–liquid partition chromatography, and no vinyl ether. It was concluded that this material was the cyclic acetal of glycerophosphorylethanolamine reported by Davenport and Dawson (1962) and Pietruszko and Gray (1962). The purified material corresponding to peak II had a molar aldehyde/phosphorus ratio of less than 0.1. It contained ethanolamine but no fatty acid or vinyl ether. After acetolysis–saponification (Thompson and Lee, 1965), the ether-soluble fraction contained glyceryl ethers as determined by gas–liquid partition chromatography of the isopropylidine derivatives (Hanahan *et al.*, 1963). The molar ratio of glyceryl ether/phosphorus calculated from the peak areas of the gas–liquid chromatogram was 0.92. After periodate oxidation (Carter *et al.*, 1961) of the glyceryl ether fraction, no isopropylidine derivative could be detected. It was concluded that this material was the 1-alkyl ether derivative of glycerophosphorylethanolamine reported by Svennerholm and Thorin (1960) and Ansell and Spanner (1961, 1963a). Chimyl, batyl, and selachyl alcohols were the major components and represented 27, 40, and 33%, respectively.

F. RECOVERY OF PHOSPHOLIPID PHOSPHORUS. In 36 complete assays of rat brain lipids at different stages of brain development to be reported elsewhere, the average recovery of phosphorus in the combined fractions was 97.5 ± 1.0%. The recovery of phosphorus

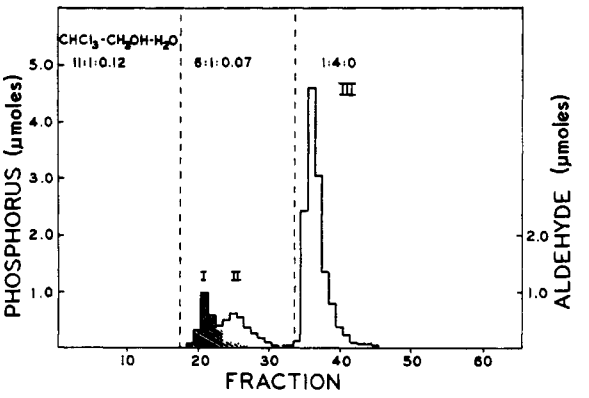

FIGURE 7: A preparative silicic acid fractionation of the chloroform-soluble mild acid hydrolysis products of rat brain lipids. A sample containing 16 μmoles of phosphorus was separated on a 10-g silicic acid–5-g Hyflo Super Cel column with the solvents indicated. Column size and solvent volumes were proportional to those described for the 2-g analytical columns described in the text. The shaded area represents the aldehyde content.

from individual anion-exchange columns and silicic acid columns was from 98 to 99%.

Evaluation of the Quantitative Analysis Procedure for Lipids Other Than Phospholipids. Ganglioside recovery in the aqueous phase after partitioning total brain lipids with chloroform–methanol–aqueous KCl was checked on the basis of the recovery of the hexosamine and sialic acid of the extract in the aqueous phase In several experiments 96–97% of the hexosamine and 95–98% of the sialic acid was recovered.

Cholesterol of lipid extracts and cholesterol added to lipid extracts were recovered in the $CHCl_3$ effluent of the Florisil column to the extent of 99%. The use of Florisil in this application is the only truly novel feature of what is an otherwise thoroughly proven assay procedure. Only traces of cholesterol ester and glycerides could be detected when this Florisil effluent was chromatographed on a silica gel thin layer plate with ethylene dichloride as a solvent (Jatzkewitz and Mehl, 1960). Glycerol assay on this fraction indicated that glyceride was present in concentrations less than 0.1 μmole/g wet weight of brain. These results are in agreement with earlier reports of the occurrence of sterol ester and glycerides in brain.

Galactosyl diglyceride was first reported in brain by Steim and Benson (1963) on the basis of the detection of galactosylglycerol in hydrolysates of brain lipids. This compound is eluted along with cerebroside and sulfatide from Florisil with chloroform–methanol (2:1). The presence of galactosylglycerol in this fraction was ascertained as follows. The fraction was free of phosphorus but contained glycerol. When the water-soluble mild alkaline methanolysis products of this fraction were chromatographed on paper, a single spot was detected. This compound cochromatographed

with authentic galactosylglycerol in the ethyl acetate–pyridine–water solvent system of Jermyn and Isherwood (1949), and the isopropyl alcohol–acetic acid–water system of Kaufman *et al.* (1965), and on AG-1 ion-exchange resin at pH 8.5. The molar ratio of hexose/glycerol of the water-soluble, mild alkaline methanolysis products was 0.97, and after acid hydrolysis only glycerol and galactose could be detected by paper chromatography in the ethyl acetate–pyridine–water solvent system. After alkaline and acid hydrolysis of the glycolipid fraction, alkyl ethers could not be detected at concentrations equivalent to 0.05 μmole/g wet weight of brain. This evidence rules out the occurrence of significant concentrations of the 1-alkyl ether analog of galactosyl diglyceride which has been reported by Norton and Brotz (1963).

Cerebroside and sulfatide recoveries were checked by adding purified brain cerebroside and sulfatide to rat brain lipid extracts. The added lipids (98% of them) were recovered in the chloroform phase of mild alkaline hydrolysates of the chloroform–methanol (2:1) effluent from Florisil columns.

Ceramide has been reported in ox brain by Rouser *et al.* (1961, 1963). Thin layer chromatography of total rat brain lipids on silica gel using chloroform–acetone (2:1) (Fujino and Zabin, 1962) as a solvent failed to show the presence of ceramide. Added ceramide could be detected at levels equivalent to 0.1 μmole/g wet weight of brain. This amount is well below the concentration reported in ox brain by Rouser *et al.* (1961, 1963).

The Reliability of the Analytical Method. A statistical analysis of the data obtained for the lipid composition of the brains from 3-day-old rats as part of a study of lipids in developing rat brains to be reported elsewhere is given in Table III. These data include duplicate assays on three separate extracts of two brains each. The concentration of cerebroside, sulfatide, sphingomyelin, inositol plasmalogen, and galactosyl diglyceride were too low in 3-day-old rats to permit significant data to be obtained. Data from the analyses on 42-day-old rats are included for these lipids. These data include duplicate assays on two extracts of two brains each. The concentration of cholesterol ester and glycerides was too low at any age to give statistically significant values. Generally, all the analyses duplicated within 5% although in a few cases the duplication is only within 10%.

TABLE III: Reliability of the Analytical Method.

Lipid[a]	μmoles/g Wet Wt	Std Dev
Phosphatidylcholine	14.72	0.31
Phosphatidylethanolamine	5.25	0.14
Phosphatidylinositol	1.21	0.042
Phosphatidylglycerol	0.119	0.009
Phosphatidylserine	2.91	0.11
Phosphatidic acid	0.142	0.004
Diphosphatidylglycerol	0.187	0.009
Phosphatidylglycerol phosphate	0.099	0.004
Diphosphoinositide	0.012	0
Triphosphoinositide	0.029	0.003
Choline plasmalogen	0.044	0.003
Ethanolamine plasmalogen	2.19	0.10
Inositol plasmalogen	0.127	0.010
Plasmalogenic acid	0.016	0.001
Ethanolamine glyceryl ether	0.18	0.01
Sphingomyelin	3.26	0.14
Cholesterol	10.7	0.35
Galactosyldiglyceride	1.46	0.06
Cerebroside	18.6	0.63
Sulfatide	3.22	0.16
Ganglioside (hexosamine)	0.31	0.016
Ganglioside (sialic acid)	0.51	0.017

[a] The level of cholesterol ester, glycerides, and serine plasmalogen was too low to give significant data.

Discussion

The analytical procedure described here is novel only to the extent that it combines and improves upon several proven techniques. The phospholipid assay combines features of the selective hydrolysis technique introduced by Dawson (1960) with the highly reproducible and sensitive anion-exchange chromatographic fractionation procedure of Lester (1963). Many of the drawbacks of Dawson's original technique have been eliminated by studying and adopting hydrolysis conditions which largely eliminate the artifacts he encountered. Also the use of silicic acid fractionation of the alkali–acid-stable products greatly decreases the possibility of error in determining sphingomyelin and phosphoethanolamine glyceryl ether. The determination of lipids other than the phospholipids is also largely based on proven analytical techniques. The application of these techniques has been facilitated by introducing an initial fractionation of the neutral and glycolipids on a Florisil column. This permits the cholesterol and cholesterol ester assays to be made without interference from phospholipids and also permits the determination of galactosyl diglyceride by selective hydrolysis. Considered as a whole and in respect to other analytical procedures now available, this technique quantitatively determines all of the known classes of lipids of brain. It is also extremely sensitive. A complete analysis of the lipids of an adult rat brain requires at most 300 mg of tissue for duplicate assays. The sensitivity is attested to by the fact that the method has led to the detection of three phospholipids which have not been previously reported in brain.

Only minor modifications are required to use this method in following the incorporation of radioisotope-labeled intermediates into brain lipids. Acid hydrolysis of the glyceryl ether cyclic acetal fraction will remove the cyclic acetal from the glycerol ether. The cerebroside and sulfatide can be separated on silicic acid (Wells

and Dittmer, 1965a) and the neutral lipids in the chloroform effluent from Florisil can be separated by one of several available methods (Barron and Hanahan, 1958; Fillerup and Mead, 1953). This brings up two major limitations of the method. It cannot give any information on the fatty acid composition of the phospholipids except sphingomyelin. It is possible to obtain this information on the glycolipids. The method also will not distinguish lyso compounds from their parent lipid. These are shortcomings that cannot be ignored; however, this method does provide a great deal of valuable information about brain lipids.

Finally, because of the acknowledged complexity of brain lipids and the demonstrated applicability of this method for brain lipids, it is reasonable to assume that it is also applicable to most other if not all other tissues. The one important limitation immediately obvious is the fact that in its present form, the method does not distinguish between mono-, di-, or triglycerides. In extending its use to other tissues or even to brains of other animals and particularly nonmammals we urge that careful and extensive characterization of fractions should be carried out to avoid misinterpretation due to the possible presence of as yet unidentified compounds.

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