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Chromatography of sphingolipids of human brain*

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SUMMARY

Sphingolipids of the brain were prepared by solvent extraction of the tissue or from mixed brain extracts subjected to mild alkaline hydrolysis. Their components were separated on silicic-acid columns. Through use of a gradient elution with a number of chloroform-methanol mixtures and pure methanol, satisfactory fractions were obtained in an 8-hour period. The elution patterns were constant and the recoveries of hexose and alkali-stable phosphorus high. Combined chemical, infrared, and paper chromatographic techniques were used for analysis. Pure galactocerebrosides were isolated and in some instances further characterized by gas-liquid chromatography of their fatty acid derivatives. Preliminary results suggest that cerebrosides containing normal acids are eluted earlier from silicic-acid columns than those containing hydroxylated acid. A ceramide was detected in the brain from a patient with multiple sclerosis and significant amounts of glucocerebrosides in the brain of an old patient. Dihydrocerebroside-like fractions and other not characterized sphingosine derivatives were found in some instances. Pure sphingomyelins were isolated from small amounts of brain tissue.

Recent improvements of the methods for separation and analysis of lipids allow more detailed studies of sphingolipids in smaller amounts of material (1 to 4) than was possible previously. Therefore it was decided to examine the sphingolipid composition of human brain with such improved techniques. The lipids from brain specimens which showed no gross abnormalities and from brains with multiple sclerosis were examined by various methods, including chromatography and chemical and infrared analyses. The studies presented here show that in addition to regularly occurring lipids, such as galactose-containing cerebrosides and sphingomyelins, more or less uncommon sphingolipids, e.g., glucose-containing cerebrosides, ceramides, and possibly dihydrocerebrosides can be found in human brain.

EXPERIMENTAL

Material. The brain specimens were obtained as soon after death as possible.[1] Gray matter was taken from the folia of the cerebellum and the cortical gray ribbon, and white matter from the underlying cortex. Mixed gray and white matter from various parts of the brain was examined separately. The specimens were weighed and then kept frozen until lipid extractions could be carried out shortly afterward.

Preparation of Sphingolipids. The sphingolipids were prepared from brain tissue by a modification of the isolation procedure of Carter *et al.* (5). Since thus isolated sphingolipids often contained contaminating glycerophosphatides, they were purified when necessary by mild alkaline hydrolysis, which effectively destroyed the contaminants (6, 7). Purified sphingolipids were also isolated "directly" from mixed brain lipids prepared by the method of Folch *et al.* (8), and then hydrolyzed in similar manner. Nonpurified sphingolipids or mixed brain lipids containing about 3 to 15 mg alkali-stable phosphorus and 50 to 250 mg of hexose were extracted twice with hexane-acetone 1/1 (v/v), and the suspension centrifuged after each extraction. The residue was dried with a nitrogen stream, suspended in 1 N KOH, and then hydrolyzed for 24 hours at 37° with shaking. The emulsion was neutralized with 5 N HCl and then acidified to pH 1.0 with hydrochloric acid and formic acid. The sphingolipids were precipitated with acetone, washed with 2% HCl-acetone 80/20 (v/v), and with acetone, and finally dried with a stream of nitrogen.

The efficiency of the preparation of nonpurified sphingolipids by the extraction procedure of Carter *et al.* (5) was examined by determination of cerebrosides

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[1] We wish to express our gratitude to Dr. Helena E. Riggs for the supply of the material.

and sphingomyelin in the isolated material, and the residues remaining in the acetone, ether, and ethanol extracts used in that preparation. Twelve experiments carried out in this manner showed that the isolated sphingolipids contained 80% to 90% of the total cerebrosides and a somewhat lesser percentage of the total sphingomyelin found in the whole extracted material. The actual sphingolipid yields were probably only slightly lower since rather small additional amounts of cerebrosides and sphingomyelin were obtained previously, upon further extraction of a sample by Carter *et al.* (5). Purification of the isolated sphingolipids by mild alkaline hydrolysis did not cause further significant losses since up to 90% of the cerebrosides and 80% sphingomyelin were recovered after the hydrolysis. About the same yields were obtained when the purified sphingolipids were prepared "directly" from mixed brain lipids by the method already described and the intermediary step of isolation of nonpurified sphingolipids was omitted. This simplified procedure thus was applied in several instances. The results of these hydrolysis experiments are in agreement with those of Kishimoto and Radin (4) and Rapport *et al.* (9), showing that mild alkaline hydrolysis effectively destroys contaminating glycerophosphatides while leaving even labile parts of the sphingolipid structure intact. Despite these findings, however, some of the principal results obtained with hydrolyzed material were duplicated on nonhydrolyzed lipids whenever sufficient quantity of brain tissue was available for both preparations.

Column Chromatography. Fractionation of sphingolipids was carried out by adsorption chromatography on silicic-acid columns essentially as described (1, 3, 10). Twenty-five to 50 g silicic acid (Baker-Reagent, Mallinckrodt 100 mesh for chromatography or silicic acid prepared in this laboratory from sodium silicate) was activated overnight at 110°. In most instances no addition of filter-aid was required. Fresh columns were prepared in glass tubes of 24 mm diameter for each fractionation. The amount of sphingolipids applied for the chromatography was limited to about 6 mg/g silicic acid.

The lipids were dissolved in small amounts of chloroform and the somewhat turbid solutions loaded onto the columns. About 500 ml of chloroform was passed through it, thus removing contaminating cholesterol, cholesterol esters, free fatty acids, and triglycerides. The elution of sphingolipids was carried out with mixtures of chloroform-methanol of gradually increasing methanol content, but strict gradient elution (3), which may lead to overlapping of components (11), was avoided. The elution started with three 50-ml portions of chloroform-methanol 95:5, 90:10, 85:15, and 80:20 (v/v), continued with 50 ml each of 14 more mixtures, which increased in methanol content in the same progression until they reached the final composition of 5:95 (v/v), and terminated with 300 ml pure methanol. More than 20 fractionations showed that the whole procedure might be carried out easily in 8 hours, that the peaks appeared regularly in almost exactly the same parts of the chromatograms, and that about 100% of the weight, 92% to 100% of the hexose, and 83% to 89% of the phosphorus loaded onto the columns could be recovered from the eluates. Some apparently low recovery figures, particularly phosphorus recovery values, may be caused by analytical errors in the determination of numerous small fractions.

Infrared Spectroscopy. Infrared spectroscopy of sphingolipids was carried out with the modified micro KBr disk technique developed in this laboratory (1, 2). Aliquots containing 100 to 200 μg of material were suspended in 50 mg solid KBr of small particle size obtained by freeze drying. The suspensions were pressed into disks of 6 mm diameter and 1 mm thickness, and the disks were examined in the condensed beam of a Beckman IR2A infrared spectrometer furnished with a beam condenser of KBr lenses. Blank disks of plain KBr were examined with each set of determinations, and base line calculations of absorption bands were carried out in the conventional way. Replicate measurements of absorption bands showed a standard error of $\pm$ 0.5%.

Quantitative infrared analysis of the lipid fractions was based on the measurement of three absorption bands, namely, the amide I band at 6.04 μ (1655 cm^{-1}), which all the sphingolipids have in common; the band at 10.3 μ (970 cm^{-1}), which is present in compounds containing a *trans* double bond; and finally the ester carbonyl band at 5.74 μ (1740 cm^{-1}), which appears in glycerophosphatide-contaminated fractions. The calibration curves of the bands at 6.04 μ, 10.3 μ, or 5.74 μ, which were derived from cerebrosides or sphingomyelins, and lecithin (isolated by chromatography), agreed closely with Beer's law.

The usefulness of the amide I band for analytical purposes was enhanced greatly by the finding of the similarity of its intensity in the two main types of sphingolipids, e.g., glycosphingosides and phosphosphingosides. Samples of 11 cerebroside and sphingomyelin preparations, which were isolated from five different brains each, showed average extinction coefficients of 1.22 and 1.29 arbitrary units, respectively. This finding allowed use of a common extinction coefficient for determination of both types of compounds. The correctness of this infrared method was further vali-

dated by the agreement of the results with chemical analysis of most of the lipid fractions.

The absorbance at 10.3 μ (970 cm^{-1}) was determined in sphingolipid fractions, in which it could be related to the presence of *trans* double bonds of a component (glycosphingoside) or to the lack of *trans* double bonds of a component (dihydroglycosphingoside) without being affected by overlapping phosphate absorptions such as occur in sphingomyelin. Calibrations of the band with standard cerebroside samples isolated from different brains agreed closely with one another and with Beer's law and showed average extinction coefficients (E = base line absorbence [10.3] per milligram cerebroside found by chemical analysis) which varied only slightly, e.g., from 0.28 to 0.30 arbitrary units. Since the extinction coefficient of a dihydrocerebroside must be zero, the relative cerebroside or dihydrocerebroside content of a mixture of the two components can be calculated from the equations:

$$\% \text{ cerebrosides} = \frac{\text{E of the unknown mixture}}{\text{E of cerebroside standard}} \times 100,$$

and

$$\% \text{ dihydrocerebroside} = 100 - \% \text{ cerebroside}.$$

The absolute values of the two components can be calculated from the "total" cerebroside figures obtained by chemical analysis or infrared measurement of the amide I band.

Measurement of the ester carbonyl band at 5.74 μ (1740 cm^{-1}) was standardized on phosphatidyl ethanolamine or lecithin isolated from brain and used for determination of contaminating glycerophosphatides. The determination of the components of binary mixtures was carried out in the conventional way (12, 13).

Paper Chromatography. Paper chromatography of the sugar components of glycosphingolipids was carried out essentially as described previously (14, 15, 16). A 1-mg sample of the lipid was hydrolyzed in a sealed tube with 2 N H_2SO_4 at 100° for 3 hours. The hydrolyzate was filtered for removal of fatty acids, neutralized with solid $BaCO_3$, and then filtered again. The filtrate was freeze-dried and the lyophilized material thus obtained dissolved in a small amount of distilled water. An aliquot of the solution was applied to strips of Whatman No. 1 paper. The chromatograms were developed in a butanol:pyridine:water system, air-dried, and finally sprayed with aniline hydrogen oxalate. Markers of pure sugars, such as glucose and galactose, were examined with each chromatogram.

Paper chromatography of sphingomyelin and contaminating glycerophosphatides was carried out by the procedure of Marinetti and Stotz (17). Replicate chromatograms were tested with ninhydrin reagent, Rhodamine 6-G, and appropriate reagents for choline. Unstained areas of other replicate strips of known R_f values were extracted with lipid solvents. The extracts were mixed with solid KBr for preparation of disks, which were examined by infrared spectroscopy. Extracts from plain strips treated in the same way were used as blanks for calculation of the infrared spectra (10).

Chemical Analysis. Chemical analysis supplemented infrared analysis and paper chromatography in searching for as many constituent radicals of the complex lipids as possible. Total lipid phosphorus was determined by the method of Sperry (18), alkali-stable phosphorus by the method of Schmidt *et al.* (19), hexose by the method of Brand and Sperry (20), phosphatidyl ethanolamine and serine by the method of Axelrod *et al.* (21), sphingosine by the methods of McKibbin and Taylor (22) and Sakagami (23), aldehydes by the method of Feulgen and Bersin (24), and total fatty acids of hydrolyzates by the method of Schmidt-Nielson (25). Nitrogen determinations were carried out by heating samples with concentrated H_2SO_4 in sealed Vycor glass tubes and subsequent nesslerization.

RESULTS

Chromatography of Brain Sphingolipids. The principal results of one fractionation with combined chemical, infrared, and weight determinations of the lipid fractions are summarized in Figure 1. The chemical data included in the illustration have been labeled to indicate the actual composition of the fractions only if it has been proved by a sufficient number of constituent radicals. General review of the chromatograms shows great constancy of the elution pattern, which allowed equating of solvent mixtures or fraction numbers used in different experiments. Comparison of the values of the weights, infrared and chemical determinations indicates the extent of agreement of the analytical data, which may be used as a suggestion of the correctness of the classification of the sphingolipid fractions. The chromatographic fractions presented in the illustrations can be divided into three groups of well-defined regularly occurring fractions of different sphingolipid composition, and several additional groups of less well-defined or less regularly occurring fractions.

Separation of Cerebrosides. The first group of fractions (Nos. 1 to 9) which was eluted with mixtures of chloroform-methanol ranging from 95:5 to 85:15

contained glycosphingosides. Some of the fractions showed a minimal phosphatide content. Greater amounts of contaminating phosphatides occurred in other fractionations in which a preceding hydrolysis step, as already described, was omitted. It is felt, however, that both procedures may be used for tentative characterization of the complex sphingolipid fractions.

Examination of the major fractions of the group (Nos. 6 to 8) shows agreement of the infrared and weight values with the chemical cerebroside figures derived from hexose determinations, thus suggesting that these major fractions were composed of rather pure glycosphingosides of the cerebroside class. The correctness of this assumption was proved further by chemical analysis of some of the fractions. Thus, fraction No. 7 of the experiment illustrated in Figure 1,

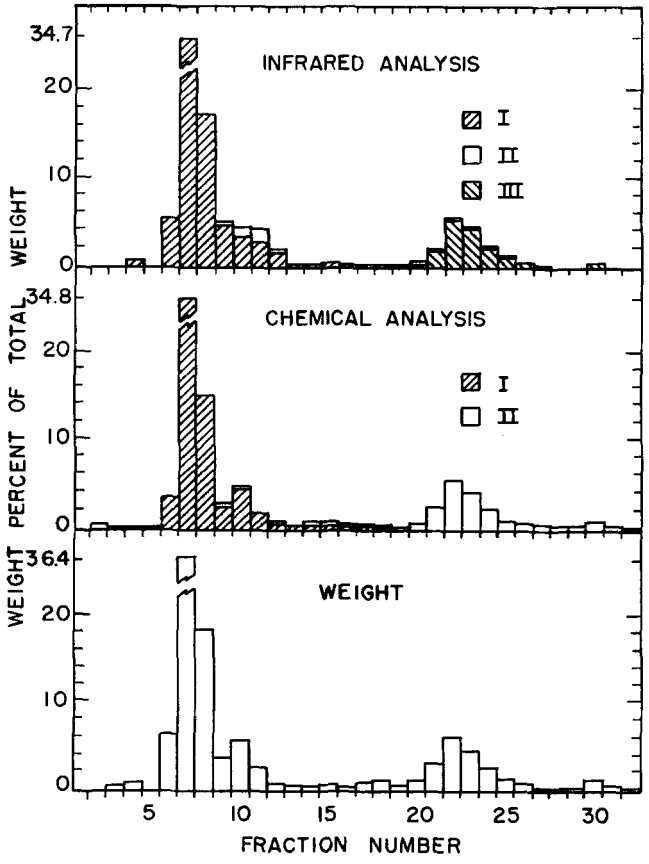

FIG. 1. Chromatographic separation of 325 mg of sphingolipids from white matter of post-mortem brain. Weight, infrared analyses (*I*, glycosphingosides; *II*, glycerophosphatides; and *III*, sphingomyelins), and chemical analysis; (*I*, cerebrosides; and *II*, phosphatides) are expressed as weight per cent of the sum of weights of all the fractions. The infrared measurements were converted into weights by use of the extinction coefficients of pure cerebrosides, sphingomyelins, and lecithins described in the text. The chemical data were converted into weights by multiplication of the analytical results by appropriate factors.

which weighed 160 mg, showed a cerebroside value of 150 mg when calculated from hexose determinations, and values of 156 mg or 160 mg when derived from sphingosine-nitrogen (2.6 mg) and fatty acid analysis (72.5 mg), respectively. These results confirm findings of Weiss (3) and preliminary reports of Schwarz *et al.* (1), and show that cerebrosides can be obtained in about as good a yield and purity by chromatography on silicic acid as by the methods of Radin *et al.* (4, 26) using Florisil and mixed ion exchange resins.

Further classification of the cerebroside fractions as phrenosine or kerasine must be deferred until gas-liquid chromatography of the fatty acids, now in progress, is completed. Separation of the normal (not hydroxylated) and hydroxy cerebroside acids carried out by the method of Kishimoto and Radin (4) showed that the first of the major cerebroside fractions (No. 7 of another fractionation) contained 76% normal and 24% hydroxy acids, while the following fraction (No. 8) was composed of 91% hydroxy and only 9% normal acids. Gas-liquid chromatography of the acid derivatives was then carried out with a Barber-Colman instrument. The normal and hydroxylated acids comprised a number of individual acids of the following chain length or retention times in minutes (t_R). The normal cerebroside acids of fraction No. 7 contained 44% acids of t_R 67 (C_{24}, lignoceric acid), 16% of t_R 48 to 17 (C_{20} and below), and 23% of t_R 9 and below, mostly stearic acid. The hydroxy acids of fraction No. 8 comprised 74% acids of t_R 86 (hydroxy lignoceric acid), two other acids amounting to 8% and 13% with t_R of 51 and 61, respectively, and two minor components with smaller t_R (even lower chain length). Further identification of these fatty acids will be carried out when additional pure standards are available. The results obtained so far agree with preliminary findings of Weiss (3) that a kerasine-containing normal fatty acid might be eluted from silicic-acid columns in front of phrenosine which contains hydroxylated acid, but stress that these two fractions are made up of a number of different fatty acids.

Galactose- and Glucose-Containing Sphingolipids. Paper chromatograms of the sugars which were isolated from the sphingolipid fractions (Nos. 4 to 18) of six different brains have been summarized in Figure 2. It may be noted from the figure that in addition to galactose, glucose, estimated as 24% of the total hexose, occurred in the sphingolipids of mixed gray and white matter of a brain of a 93-year-old. Definite but smaller amounts of glucose were found also in lipids of the white matter from a case of multiple sclerosis, while lipids of the white matter of "normal" brains contained only galactose.

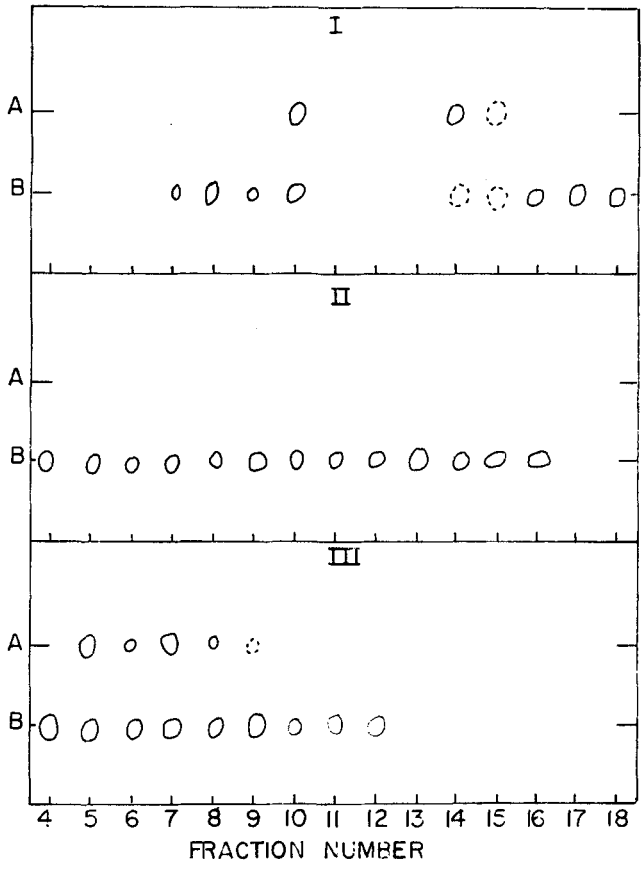

FIG. 2. Paper chromatograms of sugar preparations from the chromatographic brain sphingolipid fractions of (*I*) a case of multiple sclerosis, (*II*) five different patients who died free of nervous diseases, and (*III*) a 93-year-old. (*A*) R_f marker of glucose; (*B*) R_f marker of galactose.

Dihydrocerebrosides. The second group of fractions (Nos. 10 to 20), which was eluted with mixtures of chloroform-methanol ranging from 85:15 to 1:1 (v/v), contained glycosphingosides and small amounts of contaminating glycerophosphatides. The infrared spectra of the fractions were quite similar to those of cerebrosides, but the *trans* double bond absorption at 10.3 μ was often either absent or considerably reduced, suggesting the possible occurrence of dihydroglycosphingosides.

For further proof of this suggestion, the sphingolipid bases of this second group of fractions and of major cerebroside fractions (Nos. 7 and 8) were prepared, oxidized to aldehydes (23), and these aldehydes then examined by gas-liquid chromatography (27). The gas-liquid chromatograms showed that the aldehydes derived from the cerebroside fractions produced a major peak, probably related to the oxidation product of the sphingosine component, and a much smaller peak at about half of its retention time, probably related to small amounts of dihydrosphingosine. The chromatograms of the aldehydes from the second group of fractions, however, showed a reversed pattern, e.g., a minor peak at the longer retention time corresponding to the sphingosine and major peak at about half of the retention time, probably corresponding to the dihydrosphingosine content of the fractions. These results confirmed the suggestions from infrared spectroscopy that these fractions (Nos. 10 to 20) contained predominantly dihydroglycosphingosides.

Isolation of Sphingomyelin. The third group of fractions (Nos. 21 to 30), which was eluted with mixtures of chloroform-methanol ranging from 1:1 to 5:95 (v/v), and pure methanol, was composed mainly of rather pure phosphosphingosides. Preparations which included mild alkaline hydrolysis prior to the chromatography on silicic-acid columns yielded phosphosphingoside fractions of great purity, while isolations which omitted the hydrolysis step produced sphingosides which were often contaminated, particularly with lecithin.

The phosphosphingoside was identified as sphingomyelin by infrared spectroscopy, paper chromatography on silicic acid-impregnated paper, and chemical analysis. The infrared spectrum of such a fraction (No. 21, Fig. 1) showed the characteristic bands of pure sphingomyelin (*1*, *10*). The paper chromatograms of the fraction exhibited a single choline-positive spot at the R_f value of pure sphingomyelin. The chemical analysis of a somewhat larger fraction (No. 22, Fig. 1) showed almost negative ninhydrin reaction. The phosphorus content of 3.79%, nitrogen value of 3.2%, and sphingosine content of 30.33% found in this fraction furthermore agree with its designation as sphingomyelin. These results show that chromatography on silicic-acid columns may be used for preparation of pure sphingomyelins from relatively small amounts of brain tissue.

Other Sphingosine Derivatives. Previous studies (28, 29) showed that relatively small molecular-weight derivatives of sphingosine could be prepared by partial hydrolysis of cerebrosides and sphingomyelin, and that the ceramide, N-lignoceryl sphingosine, might be isolated even from unhydrolyzed lipids of spleen (30), liver (31), and red cells (32). There is, however, no definite evidence indicating that such sphingosine derivatives occur in brain tissue. In the present study, 21 brain sphingolipid fractionations were carried out. There were some analytical discrepancies, probably explainable in part by contaminations or analytical errors. Only two fractionations of brain lipids from cases of multiple sclerosis suggested the occurrence of ceramide-like substances.

In the first case, the fractionation of a large part of

the brain yielded a fraction No. 5, weighing 8.8 mg. It contained no hexose, not more than a trace of phosphorus (0.16%), 1.6% nitrogen, and a molar sphingosine-fatty acid ratio of 1:0.95, strongly suggesting the occurrence of a ceramide. The correctness of this suggestion was further stressed by the elution of 98% of a synthetic N-palmityl sphingosine (prepared by Dr. H. Carter and obtained from Dr. P. Kennedy) in similar chromatographic fractions (Nos. 4 and 5), and by the great similarity of the infrared spectra of both the synthetic and the brain ceramide fraction (Fig. 3). The second brain with multiple sclerosis showed a fraction No. 9 with sphingosine and fatty acid values roughly adequate for a cerebroside, but less than half of the hexose required, suggesting the occurrence of a cerebroside-ceramide mixture.

Additional sphingosine derivatives resembling mucolipids were eluted in front of the main sphingomyelin fractions (Nos. 19 to 24), particularly when lipid preparations of large amounts of gray matter were chromatographed without subjecting them to the washing procedure (8) prior to the separation. Other unidentified substances showing less phosphorus and less choline occurred at the tail of the phosphosphingoside fractions of multiple sclerosis brains. These fractions require further examination.

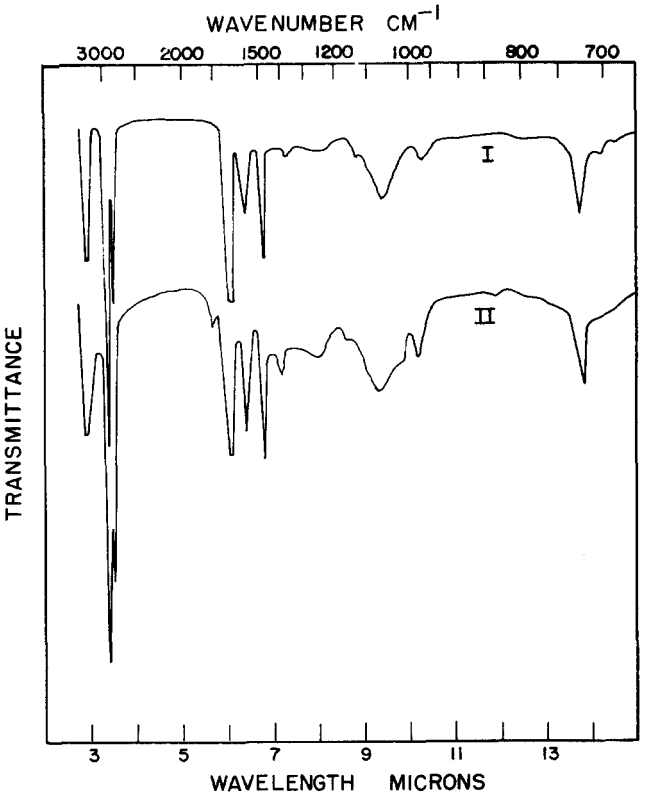

FIG. 3. Infrared spectra of (*I*) N-palmityl sphingosine and (*II*) an aliquot of the chromatographic sphingolipid fraction No. 5 from a case of multiple sclerosis (KBr disks).

DISCUSSION

The results obtained in this investigation give conclusive evidence that chromatography on silicic-acid columns, particularly when preceded by mild alkaline hydrolysis, is an effective means of isolation of glycosphingosides and phosphosphingosides. Good recoveries of hexose and alkali-stable, phosphorus-containing lipids from the hydrolyzates showed that the hydrolysis, while effectively destroying the contaminating phosphatides, did not too greatly alter the molecular structure of the sphingolipids. The principal results, furthermore, were checked on sufficiently pure nonhydrolyzed materials.

Pure glycosphingosides of the galactocerebroside class were isolated, and in part further characterized by analysis of their fatty acid components. Studies of cerebroside fatty acids of diseased human brain are in progress. Significant amounts of glucocerebrosides detected in the brain of an old patient, and smaller amounts of it found in brain with multiple sclerosis, show the importance of further examination of the sugar component of the cerebrosides. The described identification of a small ceramide fraction comprising about 1.4% of the total sphingolipids of a brain with multiple sclerosis should stimulate further search for ceramides in diseased brain tissue. The finding of a dihydroglycosphingoside in human brain, previous discovery of dihydrosphingosine in beef brain (27, 33, 34), and very recent detection of a C_{20}-sphingosine in beef and horse brain (35) stress the importance of analysis of the bases of the complex sphingolipids of the brain. Examination of the probable occurrence of sulfatides and other not yet characterized lipid components of some chromatographic fractions is in progress. The simple methods of preparation and assay described in this paper will enhance more complete characterization of sphingolipids occurring in tissues.

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