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Qualitative microanalysis and estimation of sphingolipid bases

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SUMMARY

A method is described for the identification and determination of sphingolipid long-chain bases in various animal and plant lipids. Sphingosine and related bases, isolated as a mixture from acid hydrolysates of sphingolipids, are oxidized by sodium metaperiodate and the fatty aldehyde reaction products are isolated and analyzed by gas-liquid partition chromatography. The records thus obtained reflect the composition of the sphingolipid base fraction. A preliminary survey of various tissues is reported and the types of long-chain base found for each tissue are given. Evidence is presented for the presence of a new long-chain base associated with the sphingomyelin fraction of human plasma lipids.

The standard method for the determination of sphingosine and related bases which occur in certain phospholipids, in cerebrosides, and in complex glycolipids has been that of McKibbin and Taylor (1). The method involves a nitrogen determination (micro-Kjeldahl) of a chloroform extract of long-chain base (LCB) hydrochloride and is dependent upon the chloroform insolubility of interfering nitrogenous base hydrochlorides. More recently, Sakagami has described a procedure in which fatty aldehydes, derived from sphingolipid bases by lead tetraacetate oxidation, are determined with fuchsin-sulfurous acid (2). The general reaction for the lead tetraacetate or sodium periodate oxidation of LCB is:

$$\underset{\text{OH}\quad\text{NH}_2}{\text{R—CH—CH—CH}_2\text{OH}} \xrightarrow[\text{or } \text{Pb(OCOCH}_3)_4]{\text{NaIO}_4} \text{RCHO} + \text{HCOOH} + \text{NH}_3 + \text{HCHO}$$

A preliminary report by Robins and Lowry (3) described a micromethod in which sphingomyelin and total sphingolipids were determined by colorimetric procedures.

These methods provide a quantitative assay for total LCB in lipid hydrolysates. They cannot be used, however, for the qualitative analysis of the sphingolipid bases, which generally occur as mixtures. Sphingosine is the major LCB of mammalian nerve tissue but dihydrosphingosine has also been isolated from this source (4). In addition, phytosphingosine has been reported as a constituent of plant lipids (5) and related plant bases have been described (6, 7). The known sphingolipid bases are listed in Table 1. References to the original structural studies may be found in a recent review by Carter (8).

The lack of a method for distinguishing these bases on a microscale has been a major hindrance to the study of sphingolipid metabolism. The present procedure has been developed as a microassay for the identification and determination of the sphingolipid bases in a mixture. A preliminary description of this work has been reported (9).

METHODS

Materials. Samples of triacetylsphingosine, triacetyldihydrosphingosine and 3-O-methylsphingosine were gifts from Dr. H. E. Carter. The soybean phosphatides were obtained as a gift from the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois. A sample of crude beef spinal cord cerebrosides was obtained from Dr. R. O. Brady. The aldehydes used as standards were obtained from K & K Laboratories, Long Island City, New York, and Aldrich Chemical Company, Milwaukee, Wisconsin. Samples of plasma from patients with Niemann-Pick disease or atherosclerosis were obtained through Dr. D. Fredrickson.

Isolation of Lipids. Total human plasma lipid frac-

tions were obtained by direct extraction of 100 ml. of plasma with 20 volumes of chloroform-methanol (2:1)[1] or alternatively by extraction of lyophilized plasma twice with 5 volumes of chloroform-methanol-water (12:6:1) in a Waring blendor. The white protein fluff in each case was removed by filtration and the extract was washed with one-tenth volume of water and allowed to equilibrate overnight. The bottom layer was concentrated under reduced pressure and the residue dried *in vacuo*.

A commercial powdered yeast (180 g.) was extracted for 10 minutes with 630 ml. of boiling chloroform-methanol-water (10:10:1) and the residue after filtration was re-extracted with 330 ml. of boiling chloroform-methanol-water (10:20:3). The combined extracts were concentrated to dryness *in vacuo* and the residue was dissolved in a small volume of chloroform-methanol (2:1), filtered and reconcentrated *in vacuo*.

Brain and intestinal mucosa were extracted with 20 volumes of chloroform-methanol (2:1) and the extracts were washed with water according to the procedure described by Folch *et al.* (10). The washed extracts were then concentrated to dryness *in vacuo*.

Silicic Acid Chromatography. Mallinckrodt No. 2847 silicic acid was prepared for chromatography in the following manner: The silicic acid was washed with 3N hydrochloric acid and rinsed with water until the acid was completely removed. The wet silicic acid was collected on a Buchner funnel and washed several times with alcohol and twice with acetone. The product was oven-dried and sieved. For these studies, 150 to 200 mesh silicic acid was found to be satisfactory. A slurry of 20 g. of silicic acid in about 100 ml. of chloroform-methanol (4:1) was poured into a column (1.8 cm. by 23 cm.) and allowed to settle by gravity. After washing with 50 ml. of the same solvent mixture, the column was washed with chloroform until nearly transparent. The packed height was about 18 cm. and the average holdup volume was 35 ml.

Total lipid samples were fractionated into two classes, neutral and polar lipids, by placing up to 600 mg. of lipid on the column in a small volume of chloroform. Neutral lipids were eluted with 250 ml. of chloroform and polar lipids were eluted with 400 ml. of methanol (11).

Chromatographic separations of the phosphatides were made using a modification (12) of the method described by Lea and Rhodes (13). The column was eluted with 250 ml. of chloroform, followed by chloroform-methanol mixtures as follows: 170 ml. of 9:1, 300 ml. of 4:1, 370 ml. of 1:1, and 150 ml. of 1:4. Fractions (10 ml.) were collected automatically and peaks were located by fraction weights. The chloroform-methanol (1:4) fractions, which contained all of the sphingomyelin,[2] were used for sphingolipid assay. No attempt was made to assay for cerebrosides which were eluted in earlier fractions.

TABLE 1. SPHINGOLIPID BASES

Base	Structure	Source
Sphingosine	$CH_3(CH_2)_{12}CH{=}CHCH(OH){-}CH(NH_2){-}CH_2OH$	Animal
Dihydrosphingosine	$CH_3(CH_2)_{14}CH(OH){-}CH(NH_2){-}CH_2OH$	Animal
Phytosphingosine	$CH_3(CH_2)_{13}CH(OH){-}CH(OH){-}CH(NH_2){-}CH_2OH$	Plant
Dehydrophytosphingosine	$CH_3(CH_2)_xCH{=}CH(CH_2)_{11-x}CH(OH){-}CH(OH){-}CH(NH_2){-}CH_2OH$	Plant
C_{20}-Phytosphingosine	$CH_3(CH_2)_{15}CH(OH){-}CH(OH){-}CH(NH_2){-}CH_2OH$	Plant

Lipid Hydrolysis and Isolation of the Long-chain Base Fraction. A modification of the procedure of Carter *et al.* (4) was used. The lipid sample was hydrolyzed with methanol-2N hydrochloric acid under reflux for 5 to 6 hours (80 ml. methanol and 16 ml. conc. hydrochloric acid; 8 to 10 mg. lipid per ml.). After cooling, a few drops of sulfuric acid were added and the solution

[1] All solvent ratios are v/v.

[2] The authors are grateful to Dr. D. Fredrickson for paper chromatographic identification of sphingomyelin in this peak.

was extracted three times with 2 volumes of petroleum ether (boiling range, 30°-60°C) to remove fatty esters. The methanol solution was concentrated *in vacuo* to about one-half its original volume and after chilling in an ice bath, was adjusted to pH 12 to 13 with a concentrated aqueous potassium hydroxide solution. An equal volume of water was added and the bases were extracted three times with 2 volumes of diethyl ether. The combined ether extracts were washed twice with one-tenth volume of water after which anhydrous sodium sulfate was added to dry the ether solution. The ether was evaporated at reduced pressure and the residue was dried *in vacuo*.

The crude LCB fraction was purified by silicic acid chromatography. This step was necessary to remove traces of fatty acid esters which would interfere with the gas chromatographic assay. A solution of crude LCB in 1 to 3 ml. of chloroform was placed on a small column (about 2 g. of silicic acid prepared as previously described) and esters were eluted with 20 ml. of chloroform. The bases were eluted with 30 ml. of chloroform-methanol (1:4) and the eluate was concentrated to dryness *in vacuo*. With LCB samples smaller than 10 mg. it was necessary to use solvent-washed glassware at this step and for all subsequent steps in order to avoid possible contamination. The yield of purified LCB from 100 ml. of human plasma was 3 to 5 mg.

Phytosphingosine and related bases were obtained from soybean phosphatides by the procedure described by Carter *et al.* (14). The crude LCB fraction was purified by silicic acid chromatography.

Periodate Oxidation. A 0.2 M aqueous solution of sodium metaperiodate was prepared immediately before use. The LCB was dissolved in methanol (5 mg. per ml.) in a 12 ml. centrifuge tube and one-fifth volume of the periodate solution was added. The final solution had a pH of 4 to 5 and contained approximately 15 μmole per ml. of substrate and 40 μmole per ml. of periodate. The reaction mixture stood in the dark at room temperature for 60 minutes, during which time a crystalline inorganic precipitate formed as the oxidation proceeded. No precipitate formed with a reaction blank containing no long-chain base. After an hour 2 volumes of methylene chloride and one-half volume of water were added. The mixture was shaken and centrifuged and the aqueous (upper) layer was re-extracted with 2 volumes of methylene chloride. The combined extracts were filtered through Whatman No. 1 filter paper and concentrated to dryness *in vacuo*. Benzene was added and concentration was repeated in order to remove traces of water. The crude reaction products were purified, using a small (2 g.) silicic acid column from which the aldehydes were eluted with 25 ml. of chloroform. A 10 to 20 per cent solution of aldehydes (based on the weight of purified LCB) was prepared by evaporating the chloroform eluate under a stream of nitrogen. The aldehydes were analyzed within an hour of preparation since these compounds are not highly stable. When necessary, the aldehydes can be stored overnight at −20°C in dilute chloroform solution.

Gas Chromatographic Analysis. The analyses were done using a Model 10 Barber-Colman instrument. An 8 foot, 4 mm. internal diameter glass column was packed with 60 to 80 mesh Celite 545, which was impregnated with a two-layer stationary phase. The Celite was treated with dichlorodimethylsilane and the resultant siliconized Celite was coated with a glutaric acid-diethylene glycol polyester.[3] The details of this procedure for preparing gas chromatographic column packings have been published (15). Columns prepared in this manner were very stable at 130°-200°C and provided excellent separations of saturated and unsaturated compounds in the aldehyde and fatty acid ester series.

Analyses were usually run at 180°C with an argon pressure of 25 p.s.i. and a flow rate of about 100 ml. per minute. With these conditions the slope was obtained for a semilogarithmic plot of retention time versus carbon chain length for a homologous series of authentic fatty aldehydes (Fig. 1). Although the retention times for the various aldehydes varied very little from day to day, the standard mixture was analyzed frequently as a check.

For the analysis of a mixture of aldehydes from an LCB fraction 1 μl. of the chloroform solution (equivalent to about 100 μg.) was injected into the column with a microsyringe. The gas chromatographic analysis required 45 minutes.

Compositions of LCB mixtures were calculated on the basis of the area under each aldehyde peak. Areas were determined by the product of the peak height and the width at half height. The area of the peak representing O-methylsphingosine was added to that of the peak representing sphingosine.

Catalytic Hydrogenation of Aldehydes. Hydrogenation was used to distinguish unsaturated aldehydes and determine their chain length. The aldehyde mixture after assay was evaporated to dryness, dissolved in 5 ml. of ethyl alcohol and hydrogenated with 2 mg. of 10 per cent palladium-charcoal catalyst (for 1 to 10 mg. of aldehydes) at 25°C and 1 atm. hydrogen

[3] The polyester, obtained from Dr. S. R. Lipsky, was made by Union Carbide Chemicals Co., New York, N. Y.

for 30 minutes. The mixture was filtered through a solvent-washed Celite pad and the filtrate was evaporated to dryness *in vacuo*. A 10 per cent chloroform solution of the residue was used for gas chromatographic analysis. After hydrogenation, unsaturated aldehyde peaks disappeared and were replaced by peaks representing the corresponding saturated aldehydes (see Fig. 3).

The Use of Authentic Samples of LCB. Dihydrosphingosine, isolated from an acid hydrolysate of pure triacetyldihydrosphingosine, was oxidized with periodate and analyzed as described previously. The product was chromatographically identical with hexadecanal and no other peaks were observed.

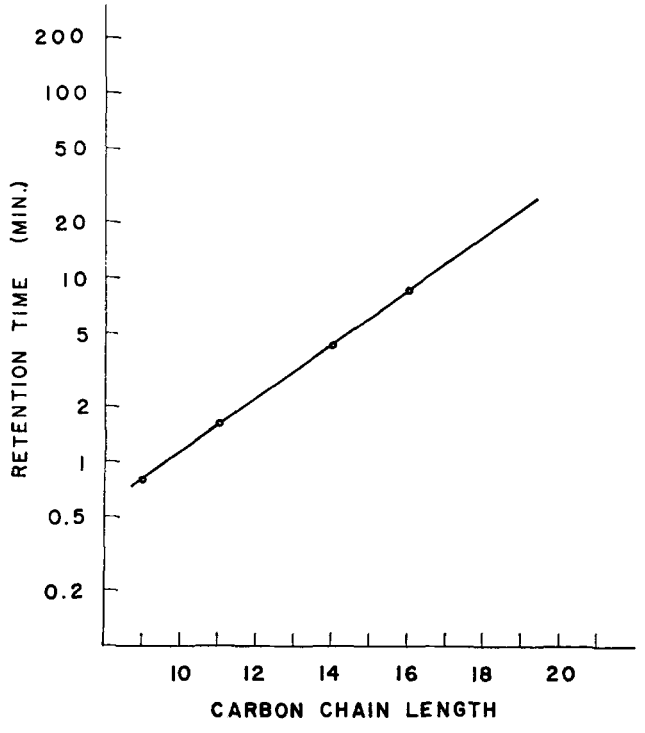

FIG. 1. Retention time of fatty aldehydes as a function of carbon chain length. Conditions of gas chromatography: glutaric acid-diethylene glycol polyester column (8 feet), 180°C, 25 p.s.i. argon.

When a sample of triacetylsphingosine was analyzed by the same procedure the assay showed a major peak (about 80 per cent), representing *trans*-2-hexadecenal, with a retention time nearly identical with that of octadecanal. After hydrogenation of the aldehyde mixture the major peak corresponded to that of hexadecanal. In addition to the major peak two minor peaks were observed which represented about 15 per cent of the total aldehyde fraction (see Fig. 2). These peaks have been identified as periodate products of 3-O-methyl ethers of sphingosine and dihydrosphingosine. The identification was based on comparisons of their retention times with those of aldehydes from authentic base samples. These ether derivatives are products of the methanol-acid hydrolysis (16). It was found that the yield of O-methyl ethers could be minimized by using methanol-hydrochloric acid rather than methanol-sulfuric acid for lipid hydrolysis.

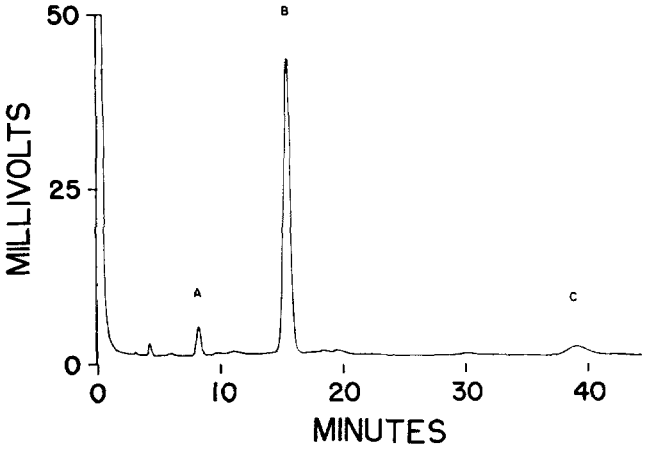

FIG. 2. Gas chromatographic analysis of LCB from bovine brain sphingolipid. Aldehyde peaks represent dihydrosphingosine (*A*), sphingosine (*B*), and O-methylsphingosine (*C*).

It is advisable to analyze solvent concentrates for the presence of high-boiling residues which might interfere with aldehyde analyses. It is usually necessary to use redistilled solvents.

RESULTS

Standard saturated aldehydes were analyzed by gas chromatography in order to determine the relationship between retention time and carbon chain length under a given set of operating conditions. A semilogarithmic plot of these data gave a straight line (Fig. 1) which was used to obtain the retention time for any saturated aldehyde in the homologous series. The conditions for hydrolysis, LCB isolation and periodate oxidation were determined with authentic samples of triacetylsphingosine and triacetyldihydrosphingosine. Periodate oxidation of dihydrosphingosine gave a product which was identical with hexadecanal, as determined by gas chromatography, and periodate oxidation of authentic sphingosine was used to establish the retention time for *trans*-2-hexadecenal.

After this procedure was established and standardized, sphingolipids from a variety of animal and plant tissues were examined. Typical analytical records are reproduced in Figures 2 to 4, which represent, respectively, LCB analyses of bovine brain, human plasma, and soybean phosphatides.

The LCB fraction of bovine brain sphingolipids was composed almost exclusively of sphingosine (94 per cent) and contained only a small amount of dihydrosphingosine.

In contrast to the relatively simple LCB composition of brain tissue, the sphingolipids of human plasma were more complex. As in brain, sphingosine was the predominant base (73 to 82 per cent) but the concentration of dihydrosphingosine was generally higher (5 to 12 per cent). In addition, every human plasma sample contained a new long-chain base to the extent of 10 to 22 per cent. Successive analyses

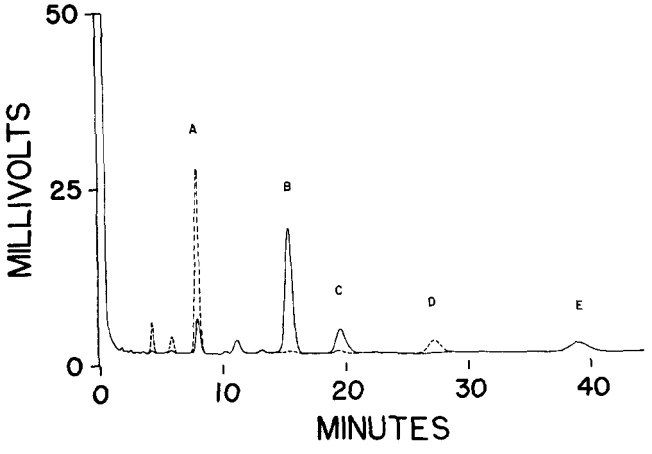

FIG. 3. Gas chromatographic analysis of LCB from human plasma sphingomyelin. Aldehyde peaks (———) represent dihydrosphingosine (*A*), sphingosine (*B*), unidentified plasma LCB (*C*), and O-methylsphingosine (*E*). Record after catalytic hydrogenation (- - - -) showing changes in retention time for unsaturated aldehydes ($B \rightarrow A$, $E \rightarrow D$).

were performed on plasma total lipids, crude phosphatides, sphingomyelin fractions from silicic acid chromatography, and sphingomyelin further purified by mild alkaline hydrolysis as described by Sribney and Kennedy (17). In every case sphingosine, dihydrosphingosine, and the unknown base were present in about the same ratio. Catalytic hydrogenation of the aldehydes from sphingomyelin LCB caused both the *trans*-2-hexadecenal and the unknown peak to disappear with a concomitant increase in the hexadecanal peak (Fig. 3).

A sample of soybean phosphatides was hydrolyzed with barium hydroxide according to the procedure of Carter *et al.* (14). Analysis of the LCB fraction showed three peaks (Fig. 4). The two minor peaks were identified as pentadecanal and hexadecanal, representing 10 per cent phytosphingosine and 5 per cent dihydrosphingosine. The major peak (B, Fig. 4) was unsaturated and yielded pentadecanal upon hydrogenation.

Analysis of crude yeast phospholipids, obtained by silicic acid chromatography, showed heptadecanal (38 per cent), pentadecanal (59 per cent), and hexadecanal (3 per cent). The heptadecanal peak confirmed the presence of C_{20} phytosphingosine in yeast as reported by Proštenik and Stanaćev (7).

Sphingomyelin fractions from plasma of patients with Niemann-Pick disease and atherosclerosis were analyzed. In neither case was the result significantly different from that of sphingomyelin from normal plasma.

The results of the various analyses are summarized in Table 2 and lipid fractionation data are given in Table 3.

DISCUSSION

The elucidation of the structures of the sphingolipid long-chain bases was the result of nearly a century of work by numerous investigators since the pioneering studies of Thudichum (18). As a result of these contributions the structures of the known sphingolipid bases have essentially been established (Table 1). There remain several problems of stereochemistry (phytosphingosine) and double bond position (dehydrophytosphingosine) yet to be solved. All these bases have in common the 2-amino and 1,3-dihydroxy groups as shown in Table 1. It is the structure of the remainder of the molecule that is different and characteristic for each base.

These differences afforded the possibility of "fingerprinting" a sphingolipid mixture. An unusual analytical problem was posed by the presence in the different bases of a double bond adjacent to the amino-dihydroxy group (sphingosine), a nonconjugated double bond (dehydrophytosphingosine), an adjacent hy-

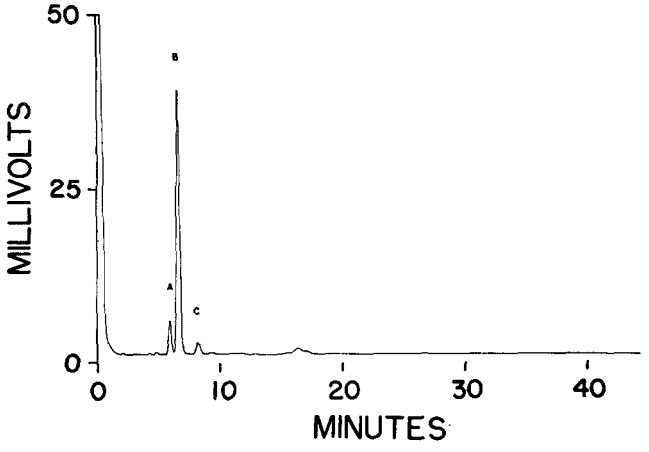

FIG. 4. Gas chromatographic analysis of LCB from soybean phosphatides. Aldehyde peaks represent phytosphingosine (*A*), dehydrophytosphingosine (*B*), and dihydrosphingosine (*C*).

TABLE 2. SPHINGOLIPID BASE COMPOSITION OF VARIOUS LIPIDS

Source	Lipid Fraction	Composition					
		Dihydrosphingosine	Sphingosine *	Unidentified Base	Phyto-sphingosine	C_{20} Phyto-sphingosine	Dehydrophyto-sphingosine
		Percentage	*Percentage*	*Percentage*	*Percentage*	*Percentage*	*Percentage*
Plasma 1	Phosphatides	5	79	16			
Plasma 2	Phosphatides	10	68	22			
Plasma 3	Phosphatides	6	82	12			
Plasma 4	Phosphatides	12	74	14			
		—	—	—			
Average †		8	76	16			
Plasma (atherosclerosis, fem.)	Sphingomyelin	11	77	12			
Plasma (Niemann-Pick disease, fem.)	Sphingomyelin	7	75	18			
Plasma (normal, fem.)	Sphingomyelin	10	73	17			
Bovine spinal cord	Cerebrosides	17	83				
Bovine intestinal mucosa	Phosphatides	3	97				
Bovine brain 1	Phosphatides	6	94				
Bovine brain 2	Phosphatides	7	93				
Yeast	Phosphatides	3			59	38	
Soybean 1	Phosphatides	6			10		84
Soybean 2	Phosphatides	5			10		85

* Figures represent the sum of the sphingosine and 3-O-methylsphingosine peaks.
† Plasmas, obtained from male donors, were A-positive (1-2) and A-negative (3-4) types.

droxyl group (phytosphingosine), and the absence of an additional functional group (dihydrosphingosine). This does not include the additional possibilities of chain length isomers and combinations of functional groups. Gas-liquid partition chromatography was the logical choice as a method for the microdetermination of a mixture of closely related materials. Since the sphingolipid bases were polar and nonvolatile, it was necessary to convert them to volatile derivatives, retaining the structural features characteristic for each base. Periodate oxidation of the sphingolipid bases satisfied these requirements and provided, as well, a simple and quantitative technique. The fatty aldehydes produced by periodate oxidation were easily analyzed by gas chromatography.

In order to separate saturated aldehydes from unsaturated aldehydes of the same chain length, a polar stationary phase was necessary. The glutaric acid-diethylene glycol polyester described by Lipsky *et al.* (19) was excellent in this respect, providing a separation factor [4] of 1.87 for hexadecanal and *trans*-2-hexadecenal.

It was convenient in these analyses to use that amount of tissue from which several milligrams of LCB could be obtained. For example, 100 ml. of human plasma, containing about 10 mg. of mixed sphingomyelins, was sufficient for a complete analysis. In the case of brain, enough sphingolipid was provided by 1 to 2 g. of fresh tissue. It will be possible to extend the method to the analysis of much smaller quantities of tissue. For example, the periodate oxidation may be carried out in a sealed capillary containing 10 μg.

[4] Separation factor is defined as the ratio of retention times for any two components.

of LCB and the entire reaction mixture analyzed, using the capillary injection system described by Bowman and Karmen (20). For this method, 1 to 2 ml. of human plasma or 10 to 20 mg. of brain tissue would be required.

As a result of a partial survey of animal and plant lipids, it is apparent that there are both qualitative and quantitative differences in tissue sphingolipids within a species, as shown in the case of bovine tissue analyses (Table 2). In addition, the sphingolipids from various sources appear to be remarkably different and perhaps characteristic, at least on the basis

TABLE 3. FRACTIONATION OF TISSUE LIPIDS

Tissue	Weight	Total Lipid	Crude Phosphatides	LCB Fraction
	g.	*g.*	*mg.*	*mg.*
Plasma 1	8.6 *	0.41	168	2.6
Plasma 2	8.2	0.40	141	3.4
Plasma 3	9.5	0.49	185	2.3
Plasma 4	8.6	0.44	173	<1.0
Plasma (normal, fem.)	8.9	0.61	29 †	1.7
Plasma (atherosclerosis, fem.)	4.0	0.56	28 †	1.3
Plasma (Niemann-Pick disease, fem.)	— ‡	0.57	21 †	8.4 §
Bovine spinal cord			594	85.0 §
Bovine intestinal mucosa	70.0 ‖	1.0	635	<1.0
Bovine brain 1	20.0	1.39	934	40.2
Bovine brain 2	20.0	1.34	1158	48.8
Yeast		14.0	460	4.0
Soybean			236	5.5

* In the plasma experiments, weights represent 100 ml. lyophilized plasma.

† Figures represent the sphingomyelin fractions obtained by silicic acid chromatography.

‡ In this case 90 ml. of plasma were extracted directly.

§ Crude fraction before silicic acid chromatography.

‖ Fresh tissue weights in mucosa and brain experiments.

of the limited data obtained. The interpretation of these data is difficult because of the almost complete lack of knowledge of the functions and intermediary metabolism of this class of lipids.

In the case of plant tissue the characteristic phytosphingosine and related bases are easily recognized since periodate oxidation liberates aldehydes containing an odd number of carbon atoms, e.g., phytosphingosine yields pentadecanal. The major component of the sphingolipids from soybean phosphatides was unsaturated and the chain length was determined by hydrogenation of the aldehyde. The results indicate that this base is dehydrophytosphingosine, which has been isolated in Carter's laboratory (6). Small quantities of phytosphingosine and dihydrosphingosine are also present in soybean phosphatides. This finding is of particular interest since dihydrosphingosine has previously been considered to occur only in animal tissue.

Analysis of crude yeast phosphatides confirms the presence of phytosphingosine and the C_{20} isomer of phytosphingosine as reported by Carter *et al.* (5) and Prošteník and Stanaćev (7), respectively. Dihydrosphingosine is again a minor constituent as in the case of soybean. The method has been applied to the analysis of human plasma sphingolipids. As expected, sphingosine is the major base component and smaller amounts of dihydrosphingosine have been found. In addition, a significant proportion of a new long-chain base is present. These three bases occurred in a similar ratio in every plasma which has been examined, including plasma from a patient with Niemann-Pick disease and a patient with atherosclerosis. The evidence obtained so far suggests that the unknown compound may be a dehydrosphingosine, and studies are currently in progress to isolate and identify the intact base.

The development of this method offers a powerful tool for investigating sphingolipid metabolism. A combination of long-chain base and fatty acid analyses, which may easily be obtained using the same gas chromatography column, provides a method for studying two variables within a mixture of sphingolipids. Thus a fresh approach may be made to the lipidoses in which sphingolipid metabolism is altered, such as Niemann-Pick, Gaucher, and Tay-Sachs diseases.

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