Phytosphingosines and Branched Sphingosines in Kidney*

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ABSTRACT: The sphingolipids of beef and rat kidney have been isolated and separated into cerebroside and spingomyelin fractions. The long-chain base mixture obtained from the cerebrosides, both beef and rat, contained a substantial amount of phytosphingosine in addition to sphingosine and dihydrosphingosine. Kidney tissue thus is unique in the simultaneous presence of all three bases. The sphingomyelin fractions of rat and beef contained no detectable phytosphingosine. However, the sphingomyelin fraction from beef, but not from rat, contained a branched chain sphingosine (13.4% of total long-chain base). Its structure was determined to be Δ^4-16-methyl-C_{17}-sphingosine. This is the first report of a branched-chain base in mammalian tissue, although branched long-chain bases have also been found in protista (Carter, H. E., Gaver, R. C., and Yu, R. K. (1966), *Biochem. Biophys. Res. Commun. 22*, 316; Carter, H. E., and Gaver, R. (1968), *Biochem Biophys. Res. Commun. 29*, 886). A crude sphingomyelin fraction from beef blood also contained this branched long-chain base, which in conjunction with the previous observations raises the question as to whether protista of the rumen may be the source of the branched long-chain base in beef kidney.

Phytosphingosines constitute the major long-chain base type in macroplants (Carter *et al.*, 1954) and have been found also in protozoa (Taketomi, 1961; Carter *et al.*, 1966), yeasts (Wickerham and Stodola, 1960), and fungi (Zellner, 1911). However, it is only recently that these bases have been detected in mammalian tissues. Karlsson (1964) reported the presence of C_{18}- and C_{20}-phytosphingosines in human kidney cerebrosides. More recently, Michalec and Kolman (1966) have found C_{20}-phytosphingosine in addition to the C_{18} homolog in kidney cerebrosides.

These findings prompted us to study the occurrence of these bases in kidney tissue of other mammals (rat and beef) and to determine to what extent these bases are localized in the various types of sphingolipids. To this end, the total lipids were extracted from fresh beef and rat kidney. Mild alkaline hydrolysis of these lipids gave a crude sphingolipid mixture which was separated into cerebroside and sphingomyelin fractions. Sphingomyelin represented the major mild alkali-stable component of both beef and rat kidney (11–12% of the total lipid). The yields and relevant analytical data are summarized in Tables I–III.

The cerebroside fraction from beef kidney was purified over DEAE-cellulose. After hydrolysis with methanolic HCl long-chain base and fatty acid fractions were separated and characterized by thin-layer chromatography and gas–liquid partition chromatography. Thin-layer chromatography showed major spots for sphingosine and phytosphingosine with a less intense spot for dihydrosphingosine. Analysis of the base mixture by gas–liquid partition chromatography of the trimethylsilyl (TMSi)[1] derivatives established the presence of C_{18}-sphingosine (major) and C_{18}-dihydrosphingosine (minor) as the only nonphyto bases. The phytosphingosine fraction contained mainly C_{18}-phytosphingosine together with trace amounts of C_{17}-phytosphingosine and a branched-chain C_{19}-phytosphingosine, as shown in Table IV. The fatty acids were analyzed as the methyl esters by gas–liquid partition chromatography. Hydroxy fatty acids accounted for a very high per cent (83) (Table III) of the total as was reported for human kidney cerebrosides (Makita, 1964; Martensson, 1966). The carbohydrate component consisted of glucose (77%) and galactose (23%), as determined by the relative areas of the TMSi ethers of the methyl glycosides on gas–liquid partition chromatography.

The sphingomyelin fraction from beef kidney was also purified over DEAE-cellulose. Hydrolysis gave a long-chain base and a fatty acid fraction. The latter consisted of C 22:0, C 18:0, and C 16:0 in decreasing amounts (in agreement with previous data on sphingomyelin from beef kidney (Spencer and Schaffrin, 1964). The long-chain base fraction, however, provided some unexpected results showing substantial differences, qualitative and quantitative (Table V), from the cerebroside composition. C_{16}-, C_{17}-, and C_{18}-sphingosines were found with the last accounting for about 50% of the total long-chain base. C_{16}- and C_{18}- dihydrosphingosines were present in minor amounts. However, no phytosphingosines could be detected and a novel base was found to be present in amounts (13.4% of long-chain base(s)) larger than any other except C_{18}-sphingosine. This base could not be detected in the cerebrosides. The new base in gas–liquid partition chromatography studies on a 3.8% SE-30 col-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: TMSi, trimethylsilyl; ECL, equivalent chain length; P, phytosphingosine; S, sphingosine; SH_2, dihydrosphingosine.

umn gave an equivalent chain length of 17.28 (C_{18}-dihydrosphingosine gives an equivalent chain length of 18.00), suggesting a branched-chain structure. Hydrogenation gave a base with an equivalent chain length of 17.65, corresponding to the disappearance of an allylic double bond. Both the reduced and the original base were treated with periodate followed by $NaBH_4$ giving the expected amounts of C_{16} alcohols. In model experiments, it was found possible to distinguish the straight-chain saturated alcohols from the isomeric iso and anteiso alcohols by gas–liquid partition chromatography of the TMSi derivatives. The first gives an equivalent chain length of *n*.00, the second of $(n - 1) + 0.65$, and the last of $(n - 1) + 0.75$. The saturated alcohol obtained from the sphingomyelin base gave an equivalent chain length of 15.65, thus providing strong evidence for an *i*-C_{16} alcohol structure. This conclusion was confirmed by a further study of the alcohol obtained from the non-reduced base. This substance by mass spectrometry gave a molecular weight of 240 (as expected for a monoenoic C_{16} alcohol). The unsaturated alcohol was oxidized with permanganate and the resulting fatty acid was converted to the methyl ester. The latter gave an equivalent chain length of 13.65 corresponding to an *i*-C_{14} fatty acid. These results establish the presence of a double bond in the 2 position of the alcohol corresponding to the 4 position in the original base. The mass spectrum of the methyl ester showed a peak at *m/e* 65 which would be expected from an iso methyl ester (Ryhage, 1967) and gave no peak at *m/e* 79, expected from an anteiso structure. These data establish the structure of the branched-chain base as Δ^4-16-methyl-C_{17}-sphingosine ($CH_3CH(CH_3)(CH_2)_{10}CH{=}CHCH(OH)CH(NH_2)CH_2(OH)$).

Branched long-chain bases have been reported only in protozoa. Carter *et al.* (1966) identified 19-methyl-C_{20}-phytosphingosine in *Crithidia fascieulata* and Carter and Gaver (1968) identified 15-methyl-C_{16}-sphingosine and 17-methyl-C_{18}-sphingosine in *Tetrahymena pyriformis*. The present base would appear to be a homolog of the last two.

Cerebroside and sphingomyelin fractions from rat kidney were treated as described for the materials from beef. Rat kidney cerebroside was found to contain 50% of C_{18}-sphingosine and 25% of C_{18}-phytosphingosine based on gas–liquid partition chromatographic analyses with internal standards. The sphingomyelin fraction contained C_{18}-sphingosine as the major component. No phytosphingosine was detected, as was the case in beef, but, in marked contrast to the results from beef kidney sphingomyelin, no trace of branched-chain base could be detected.

These findings have some interesting aspects which seem worthy of mention. The presence of phytosphingosines has been established in the kidney cerebrosides of two additional species (beef and rat), but of even more interest is the absence in the same tissues of phytosphingosine in the sphingomyelin fraction. We have observed (unpublished data) equally striking differences in the long-chain base patterns of the glyco- and phosphosphingolipids of flax. These marked differences in the long-chain base patterns of the two types of sphingolipids suggest a specificity in their biosynthesis which argues against a common long-chain base-containing intermediate. In this connection the possibility should not be overlooked that in the biosynthesis of long-chain bases the condensation step involves an appropriate serine derivative yielding directly psychosine (from galactosylserine) or a sphingomyelin precursor (from a phosphorylated serine derivative).

TABLE I: Lipid Composition of Beef and Rat Kidney.

	Beef Kidney	Rat Kidney
Total lipids		
Per cent of dry weight	13.7	14.3
Polar lipids		
Per cent of total lipids	79.9	69.8
Polar, mild alkali-stable lipids		
Per cent of polar lipids	20.3	23.3
Per cent of total lipids	16.2	16.2

The fact that kidney contains simultaneously three major long-chain base types (sphingosines, dihydrosphingosines, and phytosphingosines) makes it a unique tissue (as yet). Thus, kidney might be an especially promising tissue with which to confirm current speculations as to the biosynthetic relationships of the three types (dihydrosphingosine, phytosphingosine, and sphingosine).

It may well be asked whether the phytosphingosine and the branched sphingosine are synthesized by the kidney or are transferred there from the blood. This question seems especially relevant in the case of the branched-chain sphingosine whose presence in beef kidney and absence in rat may reflect synthesis by rumen protista of the former animal. Such an argument would be less relevant in the case of phytosphingosine, which occurs in both beef and rat kidneys. In an attempt to gain further information on this point, lipids were extracted from 50 g of dry beef blood and analyzed for long-chain base. No phytosphingosine could be detected, but branched-chain sphingosine was found both in the alkali-stable fraction and in a sphingomyelin-rich fraction. The absence of phytosphingosine in blood suggests that phytosphingosines may be synthesized in the kidney; studies are in progress on this point.

Methods and Materials

Beef Kidney. EXTRACTION AND PURIFICATION OF SPHINGOLIPIDS. Fresh tissue was homogenized in a Waring Blendor and immediately frozen and lyophilized. The lipids were extracted according to Folch *et al.* (1951), from 50 g of dry tissue. Total lipids (6.824 g) were applied to an 80-g silicic acid column (Unisil, Clarkson Chemical Co., Williamsport, Pa.) and "neutral" lipids eluted with 1600 ml of chloroform. "Polar" lipids were eluted with an equal volume of methanol. The "polar" fraction (5.477 g) was dissolved in chloroform–methanol (2:1) to a final concentration of 40 mg/ml and subjected

TABLE II: Fractionation of Polar, Mild Alkali-Stable (PMAS) Lipids from Beef and Rat Kidney.

Fraction	Eluting Solvent	Amt (% of PMAS)		Wt of LCB[a] % of PMAS		Wt of LCB[a] % of Total LCB	
		Beef	Rat	Beef	Rat	Beef	Rat
I	Chloroform–methanol (97:3)	12.9	3.9	8.0	29.2	3.9	3.8
II	Chloroform–methanol (80:20)		12.0		24.2		9.6
II-A	Acetone–methanol (90:10)	8.3		29.3		9.0	
II-B	Acetone–methanol (90:10)	3.5		13.7		1.8	
III	Chloroform–methanol (40:60)	69.8	77.6	32.4	33.0	84.1	84.2
IV	Methanol	5.5	6.5	6.4	11.3	1.3	2.4

[a] LCB = long-chain base.

TABLE III: Fatty Acid Composition of Beef Kidney Cerebrosides.

ECL[a]	Area %	Tentative Identification	ECL	Area %	Tentative Identification
16.00	2.7	16:0	21.30	5.3	20α-OH
17.20	3.0	16α-OH	22.0	6.6	22:0
17.70	Trace	18 unsaturated	22.30	2.3	21α-OH
18.00	1.2	18:0	23.00	2.2	23:0
19.20	2.3	18α-OH	23.30	33.6	22α-OH
20.00	1.2	20:0	24.00	3.3	24:0
20.20	Trace	19α-OH	24.30	19.9	23α-OH
20.70	Trace	21 unsaturated	25.20	16.4	24α-OH
21.00	Trace	21:0			

[a] ECL = equivalent chain length.

to mild alkaline hydrolysis by adding an equal volume of 1 N CH_3OH–NaOH. The solution was left at room temperature for 30 min. After neutralization and Folch partition, the lower phase was taken to dryness, dissolved in a small volume of chloroform, and applied to a 50-g silicic acid column. "Neutral" lipids were eluted with 1000 ml of chloroform followed by an equal volume of methanol which removed the polar, mild alkali-stable lipids (1.110 g). This fraction (800 mg), which contained cerebrosides and sphingomyelins, was applied to an 85-g silicic acid column. The column was first eluted with 1800 ml of chloroform–methanol (97:3). Next, cerebrosides were eluted with acetone–methanol (90:10) as two fractions (II-A, 300 ml, and II-B, 1300 ml). Finally sphingomyelins were eluted with 1600 ml of chloroform–methanol (40:60).

Fraction II-A (65 mg), which contained most of the cerebrosides, was further purified on a 9.0-g DEAE-cellulose column by eluting with chloroform-methanol (90:10), as described by Rouser *et al.* (1963). The purified material moved as a single spot on thin-layer chromatography in a chloroform–methanol–H_2O (100:42:6) system, in the same region of a mixture of standard brain and spinal cord cerebrosides. The infrared spectrum was also very similar to this standard.

The sphingomyelin-containing fraction was also further purified on a 12.0-g DEAE-cellulose column by eluting with chloroform–methanol (90:10). This fraction ran on the former thin-layer chromatography system as a single spot in the same region as standard brain and spinal cord sphingomyelin. The infrared spectrum was in close agreement with the standard and the nitrogen to phosphorus ratio was 1.99:1.

CHARACTERIZATION OF CEREBROSIDES. For sugar, fatty acid, and long-chain base(s) analyses of the cerebrosides, 1-mg samples were subjected to methanolysis in 1 ml of 1 N MeOH–HCl (10 M in H_2O) at 75–80° for 16 hr as described by Carter and Gaver (1967). The hydrolysate was taken to dryness under nitrogen; then 1.8 ml of methanol and 0.2 ml of water were added and the fatty acid methyl esters were removed by three extractions with 4 ml of low-boiling (bp 38.5–48.5°) petroleum ether as described by Kates (1964). The lower layer, containing the sugars and long-chain base(s), was taken to dryness under nitrogen and 0.5 ml of aqueous 5 N NaOH and 1.5 ml of water were added to the residue. The free

TABLE IV: Phytosphingosines of Beef Kidney Cerebrosides.

Identification	LCB[a]		Alcohol		LCB Reduced		Alcohol Reduced	
	ECL[b]	Area %	ECL	Area %	ECL	Area %	ECL	Area %
$C_{17}P$	18.45	3.1	14.00	2.0	18.45	2.0	14.00	2.0
$C_{18}P$	18.45	53.3	15.00	59.1	19.45	63.0	15.00	66.4
C_{19}br-P[c]	20.10	3.2	15.70	4.2	20.10	3.0	15.70	2.0

[a] See Table II, footnote *a*. [b] See Table III, footnote *a*. [c] br = branched.

TABLE V: Long-Chain Bases of Beef Kidney Sphingomyelin.

Identificaton	LCB[a]		Alcohol		LCB Reduced		Alcohol Reduced	
	ECL[b]	Area %	ECL	Area %	ECL	Area %	ECL	Area %
C_{16}-S	15.65	4.8						
C_{16}-SH_2	16.00	2.6	14.00	5.0	16.00	8.0	14.00	5.1
C_{17}-S	16.65	11.9	15.00	12.8	17.00	10.4	15.00	11.0
C_{18}br-S[c]	17.28	13.4	15.65	11.4	17.65	9.3	15.65	9.6
3-O-methyl-C_{18}-S	17.10	6.7						
C_{18}-S	17.65	50.0						
C_{18}-SH_2	18.00	4.2	16.00	60.9	18.00	64.5	16.00	69.0

[a] See Table II, footnote *a*. [b] See Table III, footnote *a*. [c] See Table IV, footnote *c*.

long-chain bases were removed by three extractions with 4 ml of diethyl ether, the sugars remaining in the lower layer. After methylation of the fatty acids with diazomethane, they were analyzed by gas–liquid partition chromatography on a 3.8% SE-30 column, as described by Carter and Gaver (1968). Qualitative sugar analysis was done as TMSi derivatives on a 3.8% SE-30 column. Colorimetric sugar analyses were done on the total hydrolysate, as described by Svennerholm (1956), and found to be 20% by weight. Long-chain base nitrogen was determined colorimetrically by the method of Lauter and Trams (1962).

Two thin-layer chromatography systems were used to tentatively identify the long-chain base(s): chloroform–methanol–H_2O (100:42:6) and cholroform–methanol–NH_4OH (100:25:2.5). Long-chain base(s) as TMSi derivatives were further identified by gas–liquid partition chromatography on 3.8% SE-30 columns as described by Carter and Gaver (1967). To establish which bases were unsaturated, 4.2 mg of cerebrosides was dissolved with 2 ml of absolute ethanol in a test tube sealed with a three-way stopcock. Using approximately 20 mg of 5% palladium on charcoal as catalyst (Baker and Co., Newark, N. J.) the samples were hydrogenated for 2 hr at 15 psi, with occasional evacuation of the tube. The samples were recovered by filtration through a sintered-glass funnel with successive washings of the catalyst with 20 ml of absolute ethanol and 10-ml fractions of chloroform, methanol, and finally chloroform–methanol (2:1). After methanolysis and extraction as described previously, the reduced bases were characterized by gas–liquid partition chromatography.

Periodate oxidation was performed as described by Sweeley and Moscatelli (1959). For this, the long-chain base(s) extracted from 1 mg of cerebrosides was taken to dryness and dissolved in 0.6 ml of chloroform–methanol (1:1); 0.1 ml of freshly prepared 0.2 M sodium metaperiodate was added and the solution was left in the dark for 90 min at room temperature. Then 1.0 ml of methanol–H_2O (1:1) and 1.3 ml of chloroform were added to give a Folch partition. The lower phase, containing the aldehydes, was taken to dryness under nitrogen. The aldehydes were reduced to alcohols by dissolving them in 0.5 ml of methanol and adding 0.5 ml of a $NaBH_4$ solution (1 g of $NaBH_4$ plus 8 drops of 1 N NaOH diluted to 10 ml with water) and after 30 min at room temperature 0.03 ml of 1 N HCl, 0.52 ml of water, 0.3 ml of methanol, and 1.6 ml of chloroform were added to give a Folch partition. The alcohols were recovered in the lower layer. This layer was taken to dryness, and after adding 0.5 ml of water the alcohols were extracted with diethyl ether and determined as TMSi derivatives on a 3.8% SE-30 column.

CHARACTERIZATION OF SPHINGOMYELINS. The same procedures used for fatty acid and long-chain base(s) analyses of cerebrosides were used for sphingomyelins with the exception that methanolysis was done in 1 N MeOH–HCl (20 M in H_2O). In addition to those methods used for characterizing the long-chain base(s) of cerebrosides, the TMSi derivative of Δ^2-14-methylpenta-

decen-1-ol was isolated from a mixture of alcohol–TMSi derivatives by preparative gas–liquid partition chromatography on a Barber–Colman Selectra system Series 5000 gas chromatograph, with a hydrogen flame detector. To determine the position of the double bond in this unsaturated alcohol, oxidation with permanganate as reported by Tinoco and Miljanich (1965) was performed. In a 3-ml conical centrifuge tube, 50 μl of redistilled acetic acid was added to approximately 100 μg of alcohol. Then 0.8 mg of finely powdered $KMnO_4$ was added and thoroughly mixed for approximately 5 min. Then, 50 μl of water and a few crystals of sodium metabisulfite were added to decolorize, followed by 20 μl of 5 N HCl and 0.4 ml of water. Fatty acids were extracted three times with 2.5 ml of petroleum ether. After methylation with diazomethane, they were identified by gas–liquid partition chromatography. Branched-chain fatty acid mixture BC-1 of Applied Science Laboratories, State College, Pa., was used as standards for methyl esters. Standard branched-chain alcohols were obtained by reducing the former mixture with $LiAlH_4$.

A gas chromatograph–mass spectrometer LKB 9000 was used for the determination of the molecular weight of Δ^2-14-methylpentadecen-1-ol and for methyl 12-methyltridecanoate. The alcohol was run on an 8% Apiezon column at 220° and the ester on a 3% OV-1 column at 190°. In both cases the acceleration voltage was 5000 V and the ionizing potential 70 eV at 60 μA of emission.

Rat Kidney. EXTRACTION AND PURIFICATION OF SPHINGOLIPIDS. Total lipids (3.572 g) were extracted from 25 g of freeze-dried tissue. After separation into "neutral" and "polar" fractions, the "polar" lipids (2.502 g) were subjected to mild alkaline hydrolysis as described for beef kidney, yielding 440 mg of polar, mild alkali-stable lipids. These were applied to a 58-g silicic acid column and a cerebroside-rich fraction eluted with 1000 ml of chloroform–methanol (80:20) followed by a sphingomyelin-rich fraction which was eluted with an equal volume of chloroform–methanol (40:60). No further purification of these fractions was undertaken.

CHARACTERIZATION OF CEREBROSIDES AND SPHINGOMYELINS. Long-chain base(s) analyses were performed on these compounds. The isolation of the free bases and the determinations as TMSi derivatives were the same as described for beef kidney long-chain bases.

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