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Biochemistry of the Sphingolipids

XIII. DETERMINATION OF THE STRUCTURE OF CEREBROSIDES FROM WHEAT FLOUR*

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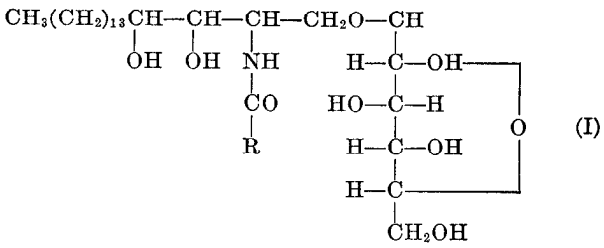
Since the initial work of Bamberger and Lansiedel in 1905 (3), numerous attempts have been made to isolate cerebrosides from such plant materials as seeds (4), fungi (5–8), and oak wood (9). The crude materials obtained were not well characterized and indeed doubt exists as to whether these preparations actually contained any cerebroside. Recently, we reported evidence (10) for the presence of a minor cerebrosidelike constituent of the glycolipid fraction of wheat flour lipid. In view of the uncertainty as to the existence of cerebrosides in plants, it seemed of interest to isolate and further characterize this material. The work presented in this communication establishes the presence in wheat flour of a mixture of cerebrosides derived from at least three different long chain bases, one of which has not been detected previously.

Cerebroside fractions were readily obtained from crude wheat flour glycolipid or from purified galactosyl-glycerol lipid fractions by an alkaline hydrolysis to destroy ester groups. These crude fractions on further purification yielded material analyzing correctly for a cerebroside and giving very similar infrared spectra. Acid hydrolysis of the purified cerebroside fraction gave glucose, a mixture of fatty acids of which the main constituent was α-hydroxystearic acid, and a long chain base fraction. These results establish the presence in wheat flour lipids of glucocerebroside(s).

In previous work with sphingolipids, we have found sphingosine and dihydrosphingosine to be present in animal but not in plant tissues; whereas phytosphingosine, the major constituent of plant sphingolipids, was not detected in animal lipids. It therefore seemed likely that the wheat cerebroside would have structure I.



(I)

Such a compound should react with 3 moles of periodate yielding pentadecanal as one of the products. The cerebroside, however, consumed only slightly more than 2 moles of periodate and little long chain aldehyde was formed. These observations made it necessary to investigate more carefully the composition of the long chain base fraction.

Preliminary attempts to obtain a homogeneous acetyl or benzoyl derivative from the long chain base fraction were unsuccessful. The cerebroside fraction showed a *trans* double bond peak in the infrared. This was abolished by catalytic hydrogenation. Acid hydrolysis of the reduced cerebroside gave a long chain base fraction which again failed to yield homogeneous derivatives. These results strongly indicated that a mixture of bases was present and in view of the well known acid lability of both sphingosine and phytosphingosine another degradative approach seemed desirable. For this purpose, a new procedure for the degradation of cerebrosides was devised (2) involving periodate cleavage of the glycosidic moiety, reduction of the dialdehyde with $NaBH_4$, and cleavage to the ceramide under very mild acidic conditions. In this way, excellent yields of pure *erythro*ceramide are obtained from phrenosin and kerasin.

Application of this procedure to unsaturated wheat flour cerebroside gave a crystalline ceramide which on alkaline hydrolysis yielded a base fraction from which pure dihydrosphingosine derivatives could be obtained readily. However, better yields of the dihydrosphingosine derivative resulted when the ceramide was reduced before hydrolysis. These results establish for the first time the presence in plant sources of a dihydrosphingosine-containing cerebroside and suggest that an unsaturated congener is also present. That this latter substance is not sphingosine is indicated by our failure to obtain the triacetyl derivative.

It should be noted that the above data do not exclude the presence of phytosphingosine (or dehydrophytosphingosine) inasmuch as these bases would have been destroyed in the periodate degradation step. In order to get a complete picture of the long chain base pattern the analytical method of Sweeley and Moscatelli (11) was applied to the mixture of bases obtained by acidic

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hydrolysis of the original cerebroside mixture. In this procedure, the long chain base is cleaved by periodate and the resulting aldehyde(s) is identified by vapor phase chromatography. Unsaturated aldehydes can be reduced to saturated aldehydes as a further means of characterization. The data on retention time given in Table I and Fig. 1 clearly show that the wheat flour cerebroside contains three major bases and one minor component. Peaks *a* and *c* are pentadecanal and hexadecanal derived, respectively, from phytosphingosine and dihydrosphingosine. Peaks *b* and *d* are converted to Peaks *a* and *c* on hydrogenation and must, therefore, represent unsaturated derivatives of phytosphingosine and dihydrosphingosine, respectively. Peak *b* is identical with that from dehydrophytosphingosine (12) for which structure II has been tentatively established:

$$CH_3(CH_2)_8CH{=}CH{-}(CH_2)_3\underset{OH}{CH}{-}\underset{OH}{CH}{-}\underset{NH_2}{CH}{-}CH_2OH \qquad (II)$$

The unsaturated dihydrosphingosine derivative (Peak *d* cannot be sphingosine as the conjugated unsaturated aldehyde derived from sphingosine has a substantially longer retention time (Peak *e*). It appears that this new base has an isolated double bond which may be located in a position similar to that in dehydrophytosphingosine.

These results establish that the cerebroside mixture from wheat flour contains the following four compounds:

$$CH_3(CH_2)_{13}\underset{OH}{CH}{-}\underset{OH}{CH}{-}\underset{NH{-}CO{-}R}{CH}{-}CH_2O{-}\text{glucose} \qquad (III)$$

$$CH_3(CH_2)_8CH{=}CH{-}(CH_2)_3{-}\underset{OH}{CH}{-}\underset{OH}{CH}{-}\underset{NH{-}CO{-}R}{CH}{-}CH_2O{-}\text{glucose} \qquad (IV)$$

$$CH_3(CH_2)_{14}\underset{OH}{CH}{-}\underset{NH{-}CO{-}R}{CH}{-}CH_2O{-}\text{glucose} \qquad (V)$$

$$CH_3(CH_2)_xCH{=}CH{-}(CH_2)_{12}{-}x\underset{OH}{CH}{-}\underset{NH{-}CO{-}R}{CH}{-}CH_2O{-}\text{glucose} \qquad (VI)$$

Compounds III, V, and VI are the major constituents with Compound IV present in small amounts. The possible presence of other types of base or of higher (C_{20}) or lower (C_{16}) homologues of the bases is not excluded. However, these, if present, represent a very small fraction of the cerebroside mixture. Further fractionation studies will be undertaken in an effort to isolate the various cerebrosides in a pure form.

The establishment of the presence of cerebrosides and of dihydrosphingosine in plant sources and the partial characterization of a heretofore undetected double bond isomer of sphingosine are new contributions from this study. It is indeed interesting that substances at one time considered to be unique components of nervous tissue are now found to occur in such a diverse source as wheat flour.

EXPERIMENTAL PROCEDURE

Isolation and Purification of Cerebrosides—As reported in a previous paper (10), the fractionation of wheat flour glycolipid on silicic acid gave a chloroform-methanol (94:6) fraction which showed a substantial amide content in the infrared spectrum. Crude cerebroside fractions were obtained from this material by mild alkaline hydrolysis to destroy ester lipids (13). In a typical experiment, 600 mg of this fraction were dissolved in 50 ml of 1 N sodium hydroxide in absolute methanol, 10 ml of water were added, and the reaction mixture was stirred at 37° for 36 hours. After dilution with the same volume of water and neutralization to pH 7 with 1 N hydrochloric acid (in ice), the suspension was extracted three times with chloroform. The combined chloroform extracts were washed with water, dried over sodium sulfate, and evaporated to dryness. The residue was dissolved in approximately 100 ml of 95% methanol and passed three times through a Dowex 2 (OH-phase) column (1.5 × 24 cm; freshly prepared). The methanolic solution, after evaporation and drying in a vacuum, gave 297 mg of a colorless powder (m.p. 159–162°; C 66.72, H 11.09, N 2.0%). The infrared spectrum

TABLE I

Retention time of aldehydes isolated after periodate oxidation of corresponding base

Base(s)	Peak (Retention time of aldehyde)				
	a	*b*	*c*	*d*	*e*
1. From wheat flour cerebrosides	6.6	7.2	8.2	9.3	
2. From wheat flour cerebrosides (hydrogenated aldehydes)	6.6		8.2		
3. Dihydrosphingosine			8.2		
4. Sphingosine					13.2
5. Phytosphingosine	6.8				
6. Dehydrophytosphingosine		7.4			
7. Mixture of bases 3, 4, 5, and 6	6.8	7.4	8.5		13.2

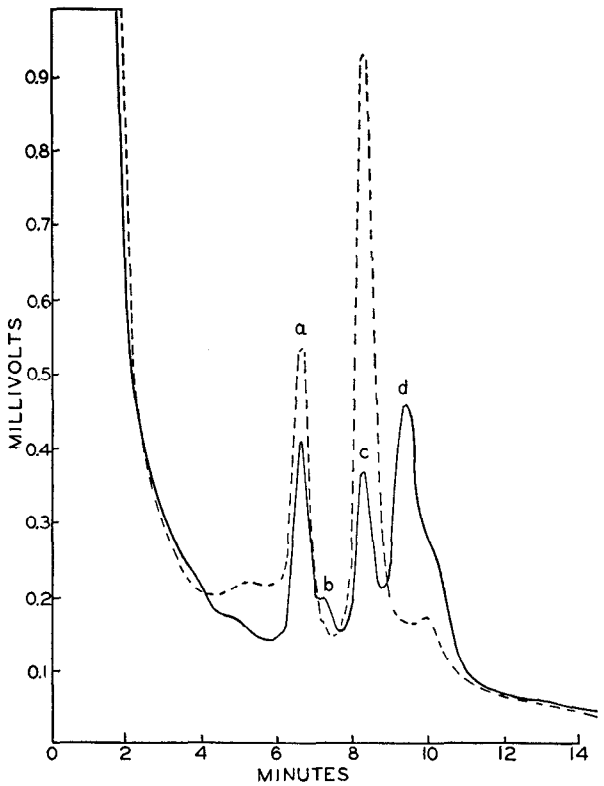

FIG. 1. Gas chromatographic analysis of long chain bases from wheat flour cerebrosides. ——, aldehyde peaks: *a*, phytosphingosine; *b*, dehydrophytosphingosine; *c*, dihydrosphingosine; and *d*, new long chain base. – – –, record after catalytic hydrogenation of unsaturated aldehydes, indicating: *a*, phytosphingosine and, *c*, dihydrosphingosine.

of this substance showed strong absorption at 3400 (hydroxyl), 1545 and 1650 (amide), and 970 cm^{-1} (*trans* double bond). The Liebermann-Burchard reaction was negative. Repeated crystallization of this material from acetone and methanol gave a white semicrystalline powder (m.p. 202–203°; $[\alpha]_D^{25} = +6.0°$ (40 mg in 2 ml of pyridine); this analyzed as C 66.06, H 10.66, N 2.20%).

Preparation of Ceramide—To a solution of 237 mg of cerebrosides in 6 ml of chloroform and 22 ml of methanol, 8 ml of 0.2 M aqueous periodic acid were added, and the reaction mixture was allowed to stand at room temperature in the dark for 19 hours. Excess periodic acid was destroyed with a few drops of ethylene glycol, water was added, and the chloroform layer was separated. The aqueous methanol layer was extracted repeatedly with chloroform. The combined chloroform extracts were washed thoroughly with water, dried, and concentrated, giving 215 mg of a colorless waxy solid. This material was dissolved in a mixture of 8 ml of chloroform, 25 ml of methanol, and 5 ml of water. The pH of the solution was adjusted to 9 with 20% aqueous sodium hydroxide, and a solution of 50 mg of $NaBH_4$ in 4 ml of aqueous 0.1 N sodium hydroxide was added. After standing 17 hours at room temperature, the solution was adjusted to pH 6 with 1 N hydrochloric acid and then acidified with 6 N hydrochloric acid to a 0.1 N solution. The reaction mixture was left at room temperature for 72 hours. Two volumes of water were added, the chloroform layer was separated, and the aqueous layer repeatedly extracted with chloroform. The combined chloroform extracts were washed with water, dried, and evaporated to dryness giving 148 mg of colorless powder (m.p. 80–95°). For purification, this material was dissolved in 2 ml of chloroform and applied to a 2-g silicic acid column. The eluate obtained with chloroform-methanol (95:5) contained 138 mg of white powder (C 73.92, H 12.02, N 2.02%). The analyses for hydroxystearoylsphingosine are (%):

$C_{36}H_{71}NO_4$ (581.94)
Calculated: C 74.30, H 12.30, N 2.41

The infrared spectrum of this material showed strong absorption at 3500 (hydroxyl), 1550 and 1675 (amide), and weaker absorption at 970 cm^{-1} (*trans* double bond). Consumption of periodate by this sample was zero.

Catalytic Hydrogenation of Ceramide—A sample of 129 mg of ceramide was dissolved in 20 ml of absolute ethanol and hydrogenated in a Parr apparatus in the presence of PtO_2 catalyst (15 mg), at room temperature. After 19 hours, the reaction was stopped, the catalyst filtered off and the clear solution evaporated to dryness in a vacuum giving 125 mg of white powder (m.p. 109–114°; C 73.77, H 12.42, N 2.25%). The analyses for hydroxystearoyldihydrosphingosine (%):

$C_{36}H_{73}NO_4$ (583.95)
Calculated: C 74.04, H 12.60, N 2.40

The infrared spectrum of this material indicated the complete elimination of the *trans* double bond.

Alkaline Hydrolysis of Ceramide—To a solution of 123 mg of ceramide in 15 ml of redistilled dioxane, 7.5 ml of water and 7.5 ml of saturated barium hydroxide solution were added, and the reaction mixture was refluxed for 24 hours in an atmosphere free of carbon dioxide. The solution was cooled, acidified to pH 6 with 6 N hydrochloric acid, and left overnight at room temperature. The combined chloroform extracts, obtained by repeated extraction of the reaction mixture, were washed once with 0.2 N hydrochloric acid, three times with water, and dried over sodium sulfate. This extract was the source for fatty acids, as will be described later. The combined aqueous fractions were made alkaline with 1 N potassium hydroxide to pH 12 and the basic material extracted repeatedly with ether. The ether extract, after washing with water and drying, was evaporated in a vacuum giving 39.7 mg of colorless powder (m.p. 73–74°). This material was ninhydrin-positive and had the following elementary composition: C 71.81, H 12.67, N 4.48%. The analyses for dihydrosphingosine are (%):

$C_{18}H_{39}NO_2$ (302.50)
Calculated: C 71.04, H 13.04, N 4.65

Periodate Oxidation Study—Periodate oxidation of the isolated base was carried out essentially by the method of Carter *et al.* (14). Determination of formic acid (calomel) gave 1.35 moles per mole of base. Formaldehyde, characterized as dimedon derivative, was formed in a yield of 0.49 mole per mole of base.

Preparation of N-Benzoyl and Tribenzoyldihydrosphingosine—To characterize the hydrogenated base, *N*-benzoyl and tribenzoyl derivatives were prepared as follows.

To a solution of 14 mg of base in 10 ml of ether, 0.2 ml of 1 N sodium hydroxide and three 0.01 ml portions of benzoyl chloride were added over a period of 30 minutes with vigorous shaking. After standing at room temperature for 5 hours, the ether was evaporated, and the residue was redissolved in 1 ml of methanol. To this solution, 0.1 ml of saturated potassium hydroxide solution was added and the reaction mixture left 15 hours at room temperature. After the addition of an equal volume of water, the suspension was repeatedly extracted with ether. The combined ether extract was washed several times with water, dried, and evaporated to dryness. The colorless powder (16 mg) thus obtained, was dissolved in chloroform and purified over silicic acid (1 g). The fraction eluted with chloroform contained 10 mg of white amorphous material (m.p. 120–122°). *N*-Benzoyldihydrosphingosine is reported to melt at 116–119° (15).

For preparation of the tribenzoyl derivative, 10 mg of the above *N*-benzoyl base was dissolved in 0.5 ml of pyridine and 0.01 ml of benzoyl chloride was added. After standing overnight at room temperature, an equal volume of cold water was added and the reaction mixture was repeatedly extracted with ether. The combined ether solution was washed with 3% sodium bicarbonate solution, then with water, dried, and evaporated to dryness, giving 17 mg of white needles (m.p. 137–145°). After recrystallization from absolute ethanol, 9.6 mg of white needles, m.p. 146–148.5°, were obtained. The melting point of this material in admixture with authentic tribenzoyldihydrosphingosine was undepressed. The elementary analyses (calculated values for tribenzoyldihydrosphingosine) were:

$C_{39}H_{51}NO_5$ (613.81)
Calculated: C 76.31, H 8.38, N 2.28
Found: C 76.29, H 8.62, N 2.31

The infrared spectrum of this material was superimposable with the spectrum of authentic tribenzoyldihydrosphingosine.

Identification of Sugar—A sample of 4.3 mg of cerebroside was hydrolyzed with 0.5 ml of 0.5 N sulfuric acid for 12 hours at 100° in a sealed tube. After cooling at room temperature, the insoluble material was filtered off. The filtrate was adjusted to pH 4.5 with Dowex 2 (HCO_3 phase) resin and then evaporated to dryness in a vacuum. The residue was redissolved in 50 μl of

water and 2 to 8 μl of this solution was applied on Whatman No. 1 filter paper for chromatography (solvent system was *iso*-propanol-acetic acid-water, 3:1:1). With an aniline-phthalate spray, a single positive spot was detected with the same R_F (0.35) as a glucose standard. (Galactose has an R_F of 0.30 in this system.) The glucose spot was also identified with the Rosenberg and Chargaff spray (16).

Identification of Fatty Acids—For identification of fatty acids, two methods were used.

(*a*) *Acid Hydrolysis*—Hydrolysis of cerebroside with 10% methanolic sulfuric acid by the method of Carter *et al.* (15) gave the methyl esters of the fatty acids, which were hydrolyzed to the free fatty acids in the usual way. This fatty acid mixture was purified on silicic acid. In a typical experiment, 47 mg of crude fatty acid was applied on silicic acid; the fraction eluted with *n*-hexane-ether (8:2) gave 27.0 mg of solid which was recrystallized twice from ethyl acetate, giving white crystals (m.p. 93–94.5°). DL-α-Hydroxystearic acid is reported to melt at 91–92° (17). The elementary analyses were in agreement with the calculated values for hydroxystearic acid.

	$C_{18}H_{36}O_3$ (300.47)		
Calculated:	C 71.94,	H 12.08,	N.E. 300
Found:	C 72.50,	H 12.15,	N.E. 296

The infrared spectrum of this material showed a typical hydroxy fatty acid pattern.

(*b*) *Alkaline Hydrolysis*—The alkaline hydrolysis of ceramide, as described earlier, gave 77.5 mg of waxy material from 123 mg of ceramide. This substance was dissolved in 5 ml of 95% ethanol and passed through a Dowex 2 (OH-phase) column. The acidic material from this column was eluted with methanolic hydrochloric acid, giving after evaporation of solvent 45.2 mg of wax. The solution of this wax in 3 ml of chloroform was applied on 5 g of silicic acid. The fraction eluted with chloroform (32 mg) showed a typical hydroxy fatty acid infrared spectrum. The methyl ester of this material was prepared with diazomethane and reduced with lithium aluminum hydride in dioxane at room temperature for 24 hours. Purification of the reduction product on silicic acid, gave 29.1 mg of a white wax eluted from a silicic acid column with chloroform-methanol (8:2). The infrared spectrum of this material showed a very strong hydroxyl absorption at 3400 cm^{-1} and the complete absence of ester absorption in the region of 1720 to 1750 cm^{-1}. This spectrum was practically identical with the infrared spectra of long chain diols in the region of 1700 to 4000 cm^{-1} (18). It seemed probable that this substance was 1,2-dihydroxy-*n*-octadecane. To prove the presence of vicinal hydroxyls, a quantitative periodate oxidation was performed. After 16 hours at room temperature, the consumption of sodium periodate was 1.2 moles per mole of diol. These experiments gave further evidence for the original α-hydroxystearic acid structure of the major fatty acid from the cerebrosides.

Gas Chromatographic Analysis—The method described by Sweeley and Moscatelli (11) was used for this analysis. The instrument was an Aerograph model A-90-C and the column the same as that described in the above paper. The analyses were run for 30 minutes at 199°, with a helium pressure of 12 p.s.i. and a flow rate of about 30 ml per minute.

For comparison, authentic samples of long chain bases were treated in the same way and analyzed under the same conditions as the unknown mixture. Sphingosine and dihydrosphingosine were isolated from brain; phytosphingosine from corn and dehydrophytosphingosine from soybean. The procedure for the isolation of these compounds has already been described (12, 14, 15, 19). It was necessary to run standard compounds on each occasion because the retention times varied slightly from run to run.

Acidic Hydrolysis of Crude Cerebroside—For gas chromatographic analysis, the fraction of crude cerebroside (eluted from silicic acid column with chloroform-methanol (94:6), as already described above) was used. This material was composed of cerebrosides, and mono- and digalactosyl-glycerol lipids. Hydrolysis of this material with 2 N methanolic hydrochloric acid (11) gave a mixture of long chain bases which was purified by silicic acid chromatography, as described by Sweeley and Moscatelli (11). The infrared spectrum of the purified bases was very similar to that of brain sphingosine, with *trans* double bond absorption at 970 cm^{-1}. Periodate oxidation of these bases was performed in the usual way. The aldehydes obtained were dissolved in benzene and the solution analyzed by gas chromatography. The results of these analyses are given in Table I, Item 1, and in Fig. 1 (——).

Hydrogenation of Unsaturated Aldehydes—The mixture of saturated and unsaturated aldehydes was hydrogenated in alcoholic solution for 2 hours in presence of 5% palladium on charcoal, at room temperature and 1 atmospheric pressure. The catalyst was removed by filtration and the solvent was evaporated at room temperature. The residue was dissolved in benzene. The infrared spectrum of the reduced aldehydes still showed the presence of carbonyl absorption (indicating selective hydrogenation of double bonds). The result of gas chromatographic analysis of this material is shown in Table I, Item 2, and in Fig. 1 (– – – –).

SUMMARY

A cerebroside fraction has been isolated from wheat flour lipid and subjected to structural studies. The presence of dihydrosphingosine, phytosphingosine, dehydrophytosphingosine, and a new long chain base in wheat flour was established. Hydrolysis of the cerebroside mixture yielded glucose. α-Hydroxystearic acid was found to be the major acidic component of wheat flour cerebrosides.

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