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Biochemistry of the sphingolipids: XII. conversion of cerebrosides to ceramides and sphingosine; structure of Gaucher cerebroside*

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SUMMARY

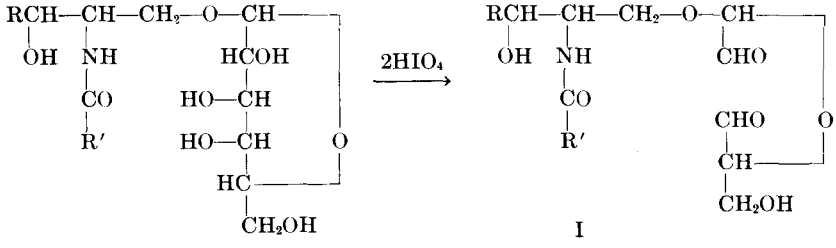
An improved procedure for the conversion of cerebroside to ceramide and sphingosine has been devised, consisting of periodate opening of the glycosidic ring, reduction with $NaBH_4$, and hydrolysis under very mild acid conditions to ceramide. The over-all yield is excellent and only the erythro isomer results. Alkaline hydrolysis of ceramide gives erythro-sphingosine in good yield. Application of this procedure to Gaucher cerebroside has shown it to contain the "normal" erythro-sphingosine.

Cerebrosides are readily available in quantity and would provide a convenient source of ceramide and sphingosine if a satisfactory procedure for removing the galactosyl moiety were available. Unfortunately, cerebrosides are moderately resistant to acid hydrolysis and, under the vigorous conditions required, yield not only "natural" erythro-sphingosine but also the threo isomer (1, 2, 3) (in aqueous solution), the 3-O-methyl ethers (4) (with methanolic acids), and other degradation products. An effective method for cleavage of cerebrosides to ceramides and sphingosine without stereochemical alterations would be valuable both for preparative purposes and for elucidation of the stereochemistry of cerebrosides.

To this end, therefore, a study of the periodate oxidation of cerebrosides was undertaken in the hope that destruction of the glycosidic ring structure would labilize the acetal bond.

A chloroform-ethanol-water system was found to be a convenient solvent for this reaction since it dissolves both cerebroside and periodic acid, with a resultant rapid, clean-cut uptake of 2 moles of periodate. The "dialdehyde" product can be isolated in excellent yield as a white, waxy solid giving acceptable analytical data. However, attempts to cleave this material under mild conditions (acidic or alkaline) were unsuccessful. Treatment of the "dialdehyde" with phenylhydrazine or cyclohexylamine (modified procedure of Barry and Mitchell [5]) gave poor yields of ceramide contaminated with nitrogenous impurities whose separation proved difficult.

The acid stability of periodate-oxidized cerebrosides is similar to that displayed by periodate oxidation products of other glycosides (6). Numerous studies have shown that these "dialdehydes" form cyclic structures (often with the inclusion of a molecule of water)



I

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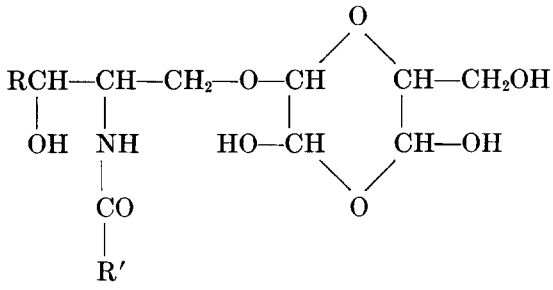
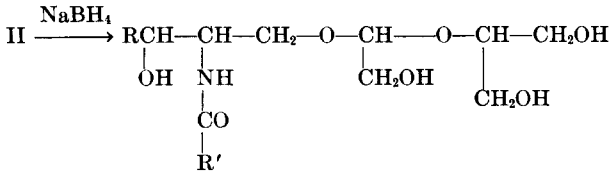
(7 to 15), which may account in part for their stability. That the cerebroside "dialdehyde" also exists in a cyclic form (II rather than I) is indicated by the absence of an aldehyde carbonyl band in the infrared spectrum. Also, the analytical data indicate the presence of an additional molecule of water.



II

The periodate-oxidized cerebroside was readily reduced to the polyol (III) by sodium borohydride (for preparative purposes it is unnecessary to isolate the "dialdehyde"). The reduction products were white solids whose analytical data supported the assigned structure



III

Hydrolysis of III with very mild acid gave ceramide in excellent yield, and alkaline hydrolysis of the latter gave erythro-sphingosine in excellent over-all yields (up to 85%) with no evidence for the production of the threo isomer. Thus these reactions provide a satisfactory preparative method for converting cerebroside to ceramide and sphingosine.

Recent reports on the structure of Gaucher cerebroside have shown that glucose is the sole carbohydrate component (16, 17); that it is attached to carbon-1 of sphingosine (17); and that the acid moiety consists mainly of a mixture of palmitic, behenic, and lignoceric acids (17). However, the stereochemistry of the sphingosine constituent and of the glycosidic bond to sphingosine has not been established. It seemed possible that the accumulation of cerebroside in Gaucher spleen might be due to the abnormal formation of the threo epimer, particularly in view of the activity of threo-sphingosine derivatives as biosynthetic precursors of sphingolipids (18, 19). Therefore a comparative study was made of the degradation of kerasin and Gaucher spleen cerebroside by the above procedure. Both cerebrosides contain normal saturated acid moieties (lignoceric in kerasin) and differ in that kerasin contains galactose as the carbohydrate constituent. If no other stereochemical difference existed, opening of the glycosidic ring should yield identical products from the two substances except for possible homologous variation in acid chain length (see structures I, II, and III). A comparison of the "dialdehyde" and "polyol" from kerasin and Gaucher cerebroside shows such close similarities that there can be no doubt as to their stereochemical identity. Furthermore, Gaucher ceramide yielded only erythro-sphingosine on further hydrolysis. These data establish that Gaucher cerebroside has the normal sphingosine structure and, furthermore, that the glucosyl moiety has the same anomeric configuration as the galactosyl constituent of kerasin. This finding is in agreement with the results of Rosenberg and Chargaff (16), based on spectroscopic evidence, that both normal and abnormal cerebrosides have the β-configuration of the glycosidic group.

EXPERIMENTAL

Preparation of Cerebrosides. Phrenosin and kerasin were obtained from a crude mixture of sphingolipids generously provided by the Wilson Co., Chicago, Illinois. Alkali-labile phospholipids and plasmalogens were first removed from the mixture (20), and the residue was then chromatographed on silicic acid-Celite®. A column 7 cm in diameter and 70 cm high was filled with a chloroform slurry of 1000 g of silicic acid (Mallinckrodt, analytical reagent, 100 mesh) and 350 g of Celite®. All bubbles of occluded air were removed, and the slurry was allowed to pack by gravity. The column was then washed with 2200 ml of methanol followed by an equal volume of chloroform. The sample (19.9 g) was dissolved in a minimum amount of hot chloroform-methanol 9/1 (v/v), and the solution, while still warm, was applied to the column. As the solution cooled and began to pass through the column, a layer of lipid was deposited in the first 2 cm of the column. One liter of chloroform-methanol 9/1 (v/v) was then applied to the column, followed by 3 liters of chloroform-methanol 8/2 (v/v). Subsequent elution was carried out with increasing amounts of methanol in chloroform. Since the first fractions eluted contained some colored matter, it was possible to complete their separation by collecting fractions manually. A crude kerasin fraction (3.2 g) followed by a crude phrenosin fraction (8.2 g) were eluted with chloroform-methanol 8/2 (v/v).

Purification of the phrenosin was accomplished by allowing a saturated solution of the crude material in low boiling petroleum ether-methanol 10/1 (v/v) to

percolate through a column of 40 g of Merck alumina which had been neutralized, washed, and dried for 24 hours at 110°. Evaporation of the effluent solution resulted in the recovery of 4.1 g of a light tan powder (impure phrenosin). White finely powdered phrenosin (2.15 g) was obtained by washing the column with a mixture of low boiling petroleum ether and methanol 1/1 (v/v). A final 0.53 g was recovered with chloroform-methanol-water 2/2/1 (v/v). The petroleum ether-methanol 1/1 (v/v) fraction was essentially pure phrenosin.

$C_{48}H_{93}NO_9$ (828.23)
Calculated: C 69.60, H 11.32, N 1.69
Found: C 67.91, H 11.17, N 1.63
$[\alpha]_D^{30} = +2.47°$ (4.86% in dry pyridine)

Hexa-acetylphrenosin was prepared from this product with acetic anhydride in pyridine (21). (Saponification equivalent: calculated 180; found 178. $[\alpha]_D^{32} = -7.32°$ [6.78% in chloroform-methanol 1/1, v/v].)

The crude kerasin was purified by recrystallization from chloroform-methanol 2/8 (v/v) followed by rechromatography on silicic acid-Celite®. A column 2 cm in diameter and 44 cm high was prepared as described above. The sample was applied as a solution in 50 ml of chloroform-methanol 95/5 (v/v). The column was first washed with 225 ml of chloroform; then chloroform-methanol 8/2 (v/v) was used to elute the pure cerebroside.

$C_{48}H_{93}NO_8$ (812.23)
Calculated: C 70.98, H 11.54, N 1.72
Found: C 70.44, H 11.05, N 1.75
$[\alpha]_D^{29} = +2.48°$ (4.89% in chloroform-methanol 1/1, v/v)
$[\alpha]_D^{30} = -2.95°$ (4.89% in dry pyridine)

An acetylated derivative of this material exhibited the following properties: Saponification equivalent: calculated 204; found 202. $[\alpha]_D^{32} = -12.9$ (7.25% in chloroform-methanol 1/1, v/v).

Gaucher cerebroside was prepared directly from a segment of a Gaucher spleen, which was made available through the generosity of Dr. R. O. Brady, of the National Institutes of Health, Bethesda, Maryland. The spleen segment was stored in the frozen state or at 4° under acetone during 20 months which passed between the time it was removed and the time at which these experiments were undertaken.

The acetone-saturated tissue (320 g) was blended, in two batches, in a Waring Blendor for 5 minutes at room temperature with a total of 1 liter of acetone. The blended mixture was allowed to stand at 4° for 4 hours and then was filtered by suction. The collected solid was suspended in 500 ml of dry acetone and stored at 4° for an additional 12 hours. At the end of this time, the solid was separated by filtration and again suspended in 500 ml of dry acetone. After standing at room temperature for 2 hours, the mixture was filtered, giving 272 g of acetone-moist tissue.

The cold acetone-extracted tissue was then subjected to continuous extraction with hot acetone in a Soxhlet apparatus for 40 hours. When cooled to room temperature, the extract yielded a light tan precipitate which was collected by centrifugation, washed four times with 15-ml portions of water, twice with acetone, and dried. The crude product weighed 10.5 g and gave an infrared spectrum which was devoid of ester absorption.

The material was further purified by chromatography on silicic acid-Celite®. A column 3.5 cm in diameter and 45 cm high was prepared according to the method described above. The sample (3.42 g) was applied to the column as a solution in 50 ml of chloroform-methanol 5/1 (v/v). One column volume (500 ml) of reagent chloroform was sufficient to remove a small amount of colored matter and all of the cerebroside (3.11 g). The purified cerebroside was a white powder which melted at 185°–187°.

$C_{46}H_{89}NO_8$ (784.18)
Calculated:[1] C 70.45, H 11.44, N 1.79
Found: C 70.27, H 11.04, N 1.71
$[\alpha]_D^{29} = -5.58°$ (5.34% in chloroform-methanol 1/1, v/v)
$[\alpha]_D^{30} = -8.94°$ (5.34% in dry pyridine)

When acetylated in the usual manner, the Gaucher cerebroside gave a derivative which melted at 94°–96°. Analytical data were in good agreement with those of a penta-acetyl derivative.

$C_{56}H_{99}NO_{13}$ (994.36)
Calculated: C 67.64, H 10.04, N 1.41, S.E., 199
Found: C 67.88, H 10.05, N 1.59, S.E., 206
$[\alpha]_D^{28} = -19.65°$ (5.9% in chloroform-methanol 1/1, v/v)
$[\alpha]_D^{28} = -25.09°$ (5.9% in dry pyridine)

Periodate Oxidation of Cerebroside. Finely powdered cerebroside (1 g) was transferred to a 100-ml volumetric flask and suspended in a mixture of 14 ml of chloroform and 51 ml of 95% ethanol. Oxidation was initiated by the addition of a measured amount (30.27 ml) of 0.2 M aqueous periodic acid. The mixture was diluted to volume with ethanol, shaken vigorously until all the lipid had dissolved, and was then allowed to stand at room temperature in the dark. Periodically, 3-ml aliquots were withdrawn from the oxidation mixture and titrated (22) against an aliquot from a blank reaction mixture to determine the extent of oxidation

[1] For purposes of comparison, the average chain length of the fatty acid constituents of the Gaucher cerebroside was assumed to be C_{22}.

(Table 1). Upon completion of the oxidation, excess reagent was reduced by the addition of 1 ml of ethylene glycol.

TABLE 1. PERIODATE OXIDATION OF CEREBROSIDES

Time	Periodic Acid Reduced	
	Kerasin	Gaucher Cerebroside
hours	*moles/mole*	*moles/mole*
1	2.03	1.35
2	1.99	1.75
3	1.99	
4		1.93
5		1.94

Although the combination of solvents used resulted in a homogenous system in which both the cerebroside and oxidizing agent remained soluble throughout the course of the reaction, any large variation in composition was sufficient to cause the mixture to separate into two phases. Thus, to recover the product, the oxidation mixture was poured into an equal volume of water; and the chloroform layer which formed was withdrawn, washed, and evaporated. Additional traces of product were obtained by further extraction of the diluted mixture with chloroform. Removal of the solvent gave the crude periodate oxidation product as a white, waxy solid.

Periodate-oxidized phrenosin: m.p. 131°–133°; $[\alpha]_D^{25} = -17.95°$ (2.95% in chloroform)

$C_{47}H_{91}NO_9$ (814.21)
Calculated: C 69.33, H 11.26, N 1.72
Found: C 68.86, H 11.09, N 1.88

Periodate-oxidized kerasin: m.p. 83°–88°

Periodate-oxidized Gaucher cerebroside: m.p. 88°–96°

An infrared spectrum of periodate-oxidized phrenosin was devoid of aldehydic carbonyl absorption indicating the existence of a cyclic "hemiacetal" structure.

Attempts to Hydrolyze Oxidized Cerebrosides with Acid and Alkali. Treatment of periodate-oxidized phrenosin (121 mg) at 37° with 0.12 N HCl in methanol for 10 hours followed by 3 hours with 0.12 N HCl in 50% aqueous methanol gave a chloroform extractable ninhydrin-negative product (117 mg), which melted in the range for known ceramides (m.p. 78°–81°) after recrystallization from acetone (C 69.58, H 11.30, N 1.85; $[\alpha]_D^{24} = -15.95°$ [2.15% in chloroform]). This product was, however, strongly aniline phthalate-positive and in other ways appeared to be practically identical to the starting material. A similar product (m.p. 80°–85°; $[\alpha]_D^{27} = -12.60°$) was obtained by refluxing periodate-oxidized phrenosin with 0.1 N HCl in 70% aqueous dioxane for 3 hours.

Attempts to obtain a ceramide or sphingosine from oxidized cerebrosides by alkaline hydrolysis were equally unsuccessful. Periodate-oxidized phrenosin (154 mg) was refluxed in 30 ml of 0.01 N NaOH in 70% aqueous dioxane for 6.5 hours in a carbon dioxide-free system. The mixture was cooled, diluted with an equal volume of water, and extracted quickly with chloroform. The extract yielded 143 mg of a white powder (m.p. 178°–182°) which gave no reaction with either aniline phthalate or ninhydrin (C 66.42, H 10.74, N 1.83 [alkaline residue]).

Periodate-oxidized phrenosin (218 mg) was also refluxed for 14 hours in 14 ml of one-quarter saturated $Ba(OH)_2$ in 50% aqueous dioxane. In this case the reaction mixture was neutralized before extraction with chloroform. Enough methanol was present during the extraction to prevent the formation of emulsions. Upon evaporation of the extracts, it was found that approximately half of the material was then chloroform-methanol insoluble and high melting (m.p. 220°). The remainder melted from 85° to 110° and was ninhydrin-positive but did not yield triacetylsphingosine when treated with acetic anhydride in pyridine.

Degradation of Periodate-Oxidized Cerebrosides with Cyclohexylamine. A modification of the method of Barry and Mitchell (5) for degrading periodate-oxidized polysaccharides was used as a possible means of obtaining crude ceramides from oxidized cerebrosides.

Periodate-oxidized phrenosin (500 mg) was suspended in a solution composed of 5 ml of cyclohexylamine and 50 ml of methanol, and the mixture was refluxed for 2 hours. Thereupon the methanol was removed under reduced pressure, and the residue was suspended in approximately 50 ml of water. This mixture was cooled on ice, slowly acidified to pH 1 with 6 N H_2SO_4, and allowed to stand at room temperature for 2 hours. The lipid was then extracted into chloroform. Enough methanol was added to prevent the formation of emulsions. The extracts were neutralized with Dowex 2 (HCO_3^-), filtered, and evaporated to dryness. The residue obtained was extracted exhaustively with hot acetone, and the extracts were concentrated, then cooled to room temperature to precipitate the crude ceramide (m.p. 78°–88°). After recrystallization from acetone, the product was a slightly tan powder (m.p. 83°–89°).

$C_{42}H_{83}NO_4$ (666.09)
Calculated: C 75.73, H 12.56, N 2.10
Found: C 73.09, H 11.95, N 3.00

This product was hydrolyzed with 1 N KOH in 90% methanol according to the method described in a following section. Acetylation of the liberated base gave crude triacetylsphingosine. Two recrystallizations from *n*-hexane yielded the pure derivative as fine white needles (m.p. 104°–106°; $[\alpha]_D^{27} = -10.1°$ [1.43% in chloroform]).

	$C_{24}H_{43}NO_5$ (425.59)
Calculated:	C 67.73, H 10.18, N 3.29
Found:	C 67.56, H 10.37, N 3.74

The yields of the products obtained by this method are summarized in Table 2. Since contaminating substances encountered with this method were also soluble in organic solvents, efficient purification of the products was difficult to accomplish by simple methods.

TABLE 2. CYCLOHEXYLAMINE DEGRADATION OF PHRENOSIN

Compound	Over-all Yield from Phrenosin
	per cent
Oxidized phrenosin	98.9
N-Cerebronylsphingosine (crude)	78.8
N-Cerebronylsphingosine (purified)	64.5
Sphingosine	64.5
Triacetylsphingosine (crude)	59.5
Triacetylsphingosine (purified)	29.3

Reduction of Periodate-Oxidized Cerebrosides. A more efficient method of degrading oxidized cerebroside was designed to take advantage of the increase in lability which periodate-oxidized glycosides exhibit upon reduction to the corresponding alcohols (23). Very finely powdered oxidized cerebroside (960 mg) was dissolved in 13 ml of chloroform. The solution was diluted with 56 ml of 95% ethanol and 20 ml of water, and then was rendered basic by the addition of 4 drops of 20% NaOH. A solution of 100 mg (fourfold excess) of $NaBH_4$ in 10 ml of 0.1 N NaOH was added with stirring, and the reaction was allowed to proceed at room temperature for 3 hours. The solution was stirred continuously and remained clear throughout this period. Reduction was terminated by careful acidification of the reaction mixture to pH 1 with methanolic HCl to decompose the excess reagent. The solution was then neutralized with 20% NaOH and poured into an equal volume of water. After standing overnight, the product-containing chloroform layer was siphoned off, and the reaction mixture was extracted twice more with chloroform. These extracts were washed twice with water and evaporated under reduced pressure to obtain the product.

Phrenosin derivative: m.p. 148°–151° (melted and resolidified, 88°–105°; $[\alpha]_D^{25} = +20.3°$ [1.42% in chloroform-methanol 1/1, v/v]).

Acetylated phrenosin derivative: m.p. 58°–60°; $[\alpha]_D^{26} = +0.226°$ (3.10% in chloroform-methanol).

	$C_{57}H_{103}NO_{13}$ (1010.40)
Calculated:	C 67.75, H 10.28, N 1.39, S.E. 202
Found:	C 67.31, H 10.23, N 1.50, S.E. 200

Kerasin derivative: m.p. 113°–118° (sintered circa 75°); $[\alpha]_D^{32} = +5.90°$ (5.13% in chloroform-methanol 1/1, v/v); $[\alpha]_D^{32} = -0.80°$ (5.09% in dry pyridine).

Acetylated kerasin derivative: m.p. 63°–65°; saponification equivalent: calculated 238; found 234.

Gaucher cerebroside derivative: m.p. 111°–114° (sintered circa 75°); $[\alpha]_D^{32} = +5.70°$ (5.14% in chloroform-methanol 1/1, v/v); $[\alpha]_D^{32} = -0.78°$ (5.08% in dry pyridine).

Acetylated Gaucher cerebroside derivative: m.p. 70°–73°; saponification equivalent: calculated 231; found 236.

Reduction of the double bond in chemically reduced periodate-oxidized kerasin and Gaucher cerebroside was effected by shaking 1% solutions of the lipids in warm absolute ethanol in an Adams apparatus for 10 hours at room temperature and 2 atmospheres of pressure in the presence of 80 mg of platinic oxide (J. Bishop and Co., Malvern, Pa.).

Kerasin derivative: m.p. 124°–129° (melted and resolidified, 85°–105°); $[\alpha]_D^{32} = +34.5°$ (5.04% in chloroform-methanol 1/1, v/v).

Gaucher cerebroside derivative: m.p. 115°–121° (melted and resolidified, 85°–105°); $[\alpha]_D^{32} = +33.5°$ (4.98% in chloroform-methanol 1/1, v/v).

Hydrolysis of Reduced Periodate-Oxidized Cerebrosides. The reduction product (830 mg) was dissolved in 60 ml of 0.1 N HCl in 90% aqueous peroxide-free tetrahydrofuran. This solution was then allowed to stand at room temperature for 24 hours. The length of the hydrolysis period was established by following the reaction optically to constant rotation. After hydrolysis, the mixture was diluted with an equal volume of water, neutralized with solid Na_2CO_3, and extracted three times with chloroform. The extracts were combined, washed twice with equal volumes of water, and then evaporated to dryness to obtain the crude product. This material was dissolved in 100 ml of hot absolute methanol and filtered to remove a small amount of an insoluble brown wax. The filtrate was concentrated under reduced pressure to approximately

25 ml, heated to dissolve any solid, then cooled to 0° to reprecipitate a white powder.

Phrenosin ceramide (cerebronyl sphingosine): m.p. 82°–86°.

	$C_{42}H_{83}NO_4$ (666.09)
Calculated:	C 75.73, H 12.56, N 2.10
Found:	C 72.60, H 11.99, N 2.30

Kerasin dihydroceramide (lignoceryl dihydrosphingosine): m.p. 95°–98°; $[\alpha]_D^{32} = +4.50°$ (2.51% in chloroform-methanol 1/1, v/v).

	$C_{42}H_{85}NO_3$ (652.11)
Calculated:	C 77.35, H 13.14, N 2.15
Found:	C 76.66, H 12.50, N 2.03

Gaucher cerebroside dihydroceramide: m.p. 94°–97°; $[\alpha]_D^{32} = +4.40°$ (2.48% in chloroform-methanol 9/1, v/v).

	$C_{40}H_{81}NO_3$ (624.06)
Calculated:	C 76.98, H 13.08, N 2.24
Found:	C 76.41, H 12.56, N 2.29

Infrared spectra of these materials showed only the absorption maxima expected of ceramides.

The aqueous solution (150 ml) obtained from the hydrolysis of the Gaucher material was filtered to remove a slight white precipitate and then was treated with a solution of 1.5 g of phenylhydrazine hydrochloride and 3 g of sodium acetate in a minimum amount of water. Within 5 minutes there was formed a bright yellow precipitate which slowly turned brown-orange upon standing at room temperature. After 15 hours the solution was filtered to obtain 91 mg of crude glyoxal phenylosazone (m.p. 156°–161°). Recrystallization from aqueous ethanol gave fine golden needles (m.p. 158°–165°).

	$C_{14}H_{14}N_4$ (238.28)
Calculated:	C 70.56, H 5.92, N 23.52
Found:	C 71.05, H 5.77, N 22.49

In contrast to the ease with which the reduced cerebroside derivatives could be cleaved with acid, treatment with 0.1 N NaOH in 70% dioxane at reflux for 6 hours gave little or no degradation.

Preparation of Ceramide from Phrenosin. For preparative purposes the three steps (periodate oxidation, $NaBH_4$ reduction, and acid hydrolysis) can be carried out without isolation of intermediate products as follows.

A sample of 1.00 g of the finely powdered colorless cerebroside (1.21 mmoles as phrenosin) was transferred to a 100-ml volumetric flask and suspended in a mixture of 14 ml of chloroform and 51 ml of 95% ethanol. Oxidation was initiated by the addition of a measured amount (30.4 ml) of 0.2 M aqueous periodic acid. The mixture was diluted to volume with ethanol, shaken vigorously until all the lipid had dissolved, and was allowed to stand at room temperature in the dark for 3 hours.

After reduction of excess periodic acid by the addition of 1 ml of ethylene glycol, 80 ml of water was added to the reaction mixture and the chloroform layer which formed was withdrawn. The water layer was extracted successively with 22-ml and 10-ml portions of chloroform to obtain additional small amounts of product. The combined chloroform extracts were then washed with 20 ml of water and with a mixture of 18 ml of methanol and 20 ml of water.

The washed chloroform extract (52 ml) was transferred to a 500-ml flask, and 224 ml of 95% ethanol and 80 ml of water were added. The solution was neutralized to pH 9 with several drops of 20% aqueous NaOH and a solution of 100 mg (2.64 mmoles) of $NaBH_4$ in 40 ml of 0.1 N NaOH was added dropwise from a pipette over a period of several minutes. The flask was then stoppered loosely and stirring was continued at room temperature for 6 hours. Reduction was terminated by careful neutralization of the reaction mixture to pH 6.8 with aqueous 6 N HCl.

The neutralized solution was further acidified with 6.7 ml of aqueous 6 N HCl in order to make the final HCl concentration of the solution 0.1 N. The solution was allowed to stand at room temperature for 24 hours. During this period the solution remained clear and colorless. The reaction mixture was diluted with water and repeatedly extracted with a mixture of chloroform and methanol 1/1 (v/v). These extracts were combined, washed with water several times, and evaporated to dryness, giving 744 mg (93% yield) of a colorless powder (m.p. 92°–94°) which was almost pure ceramide (C 75.15, H 12.34, N 2.20).

Isolation of Free Bases. Two methods were used to hydrolyze the ceramides to sphingosine (or dihydrosphingosine).

The phrenosin ceramide was refluxed for 10 hours at a concentration of 1.2% in a solution of 1 N KOH in 90% methanol. The hydrolysis mixture was diluted with an equal volume of water, allowed to stand for 12 hours at room temperature, and then extracted three times with equal volumes of ether. During the extraction enough methanol was maintained in the mixture to prevent the normally ether-insoluble base from precipitating. The ether extracts were washed until neutral, then were evaporated to recover the crude product.

The dihydroceramides from kerasin and Gaucher cerebroside were each hydrolyzed at a concentration of 0.8% in solutions consisting of peroxide-free dioxane

(50%), carbon dioxide-free water (25%), and aqueous saturated $Ba(OH)_2$ (25%). The mixtures were refluxed for 24 hours with occasional stirring to dislodge any materials which collected on the sides of the flasks. At the end of this time 3 volumes of water were added, and the solutions were filtered with suction to obtain white powders from which crude dihydrosphingosine was extracted at room temperature with four 25-ml portions of chloroform-methanol 1/1 (v/v).

Identifiable derivatives of the free bases were obtained by acetylation with acetic anhydride in pyridine (21) or benzoylation according to the method of Carter *et al.* (24).

Triacetyl-erythro-sphingosine from phrenosin: m.p. 103°–105°; $[\alpha]_D^{32} = -9.4°$ (1.3% in chloroform).

Tribenzoyl-erythro-dihydrosphingosine from kerasin: m.p. 146°–148°; $[\alpha]_D^{32} = -30.20°$ (2.34% in chloroform).

Tribenzoyl-erythro-dihydrosphingosine from Gaucher cerebroside: m.p. 146°–148°; $[\alpha]_D^{32} = -29.95°$ (2.54% in chloroform).

In order to account for maximum amounts of the kerasin and Gaucher cerebroside bases, the mother liquors which remained from the preparation of their polybenzoyl derivatives were concentrated and hydrolyzed in methanolic KOH. The bases were then reisolated and acetylated.

Triacetyl-erythro-dihydrosphingosine from kerasin: m.p. 99°–101°.

TABLE 3. STEPWISE DEGRADATION OF CEREBROSIDES

Compound	Over-all Yield from Cerebroside		
	Phrenosin	Kerasin	Gaucher Cerebroside
	per cent	*per cent*	*per cent*
Oxidized cerebroside	95.4	99.6	99.9
$NaBH_4$ reduction product	86.0	97.1	96.5
Ceramide (crude)	78.0	96.8	96.5
Ceramide (purified)		78.2	74.3
Free base (crude)	78.0	96.8	95.3
Triacetyl-erythro-sphingosine	60.8		
Tribenzoyl-erythro-dihydrosphingosine		66.2	64.9
Tribenzoyl-erythro-dihydrosphingosine plus Triacetyl-erythro-dihydrosphingosine		88.6	81.4

Triacetyl-erythro-dihydrosphingosine from Gaucher cerebroside: m.p. 98°–100°.

The yields of these derivatives are summarized in Table 3.

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