Sphingomyelin prepared with the older methods from brain and spleen yielded on acid hydrolysis palmitic, stearic, and lignoceric acids (1, 2). Merz found that in addition to these saturated acids nervonic acid is present in the sphingomyelin of brain (3).

The presence of palmitic acid in the sphingomyelin molecule of brain seemed questionable, since it was shown that sphingomyelin prepared by the usual method is a mixture of hydrolecithin (dipalmityl lecithin) and sphingomyelin.

It is the purpose of this paper to describe a procedure for the preparation of sphingomyelin free of hydrolecithin from brain and spleen, and to reinvestigate its component fatty acids. In pure sphingomyelin of brain, stearic acid, lignoceric acid, and its unsaturated compound, nervonic acid, are present. No palmitic acid was found. Stearic acid and nervonic acid are present in larger quantities than is lignoceric acid.

Sphingomyelin free of hydrolecithin was prepared from spleen according to the method previously described for the isolation of pure sphingomyelin from lung (4). The fatty acids present in the sphingomyelin of spleen are the same as those found in sphingomyelin of lung; namely, palmitic acid and lignoceric acid in about equal amounts.

Carter and coworkers (5, 6) have shown that sphingosine present in the cerebrosides molecule is partly saturated (hydrosphingosine) and partly unsaturated sphingosine. The findings of Carter prompted us to isolate sphingosine after acid hydrolysis of sphingomyelin and to investigate whether or not saturated sphingosine is, as in cerebrosides, also a constituent of the sphingomyelin molecule.

Crystalline sphingosine sulfate was isolated from pure sphingomyelin of brain and spleen. In both types of sphingomyelins the isolated sphingosine sulfates had iodine numbers which were much lower than that calculated for unsaturated sphingosine sulfate. It seems therefore justified to assume

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that the sphingosine groups not only of the cerebrosides but also of the sphingomyelins are mixtures of hydrosphingosine and sphingosine.

EXPERIMENTAL

Preparation of Sphingomyelin from Brain by Alkaline Saponification

Two batches of crude sphingomyelin from brain (16 gm. each) obtained from the ether-insoluble lipide fraction by fractionation with glacial acetic acid-acetone mixture, described in the preceding paper as Substance A (7), were separately suspended in a small amount of water and ground to a paste. To each, 320 cc. of 0.25 N sodium hydroxide were added. These suspensions were shaken at 37° for 4 to 5 days. After acidification with glacial acetic acid they were both placed in the refrigerator and left there overnight.

Despite the acidification, the insoluble sphingolipides were present partially in the form of an emulsion which could not be filtered. In order to obtain a filtrable precipitate it was necessary to add an equal volume of acetone. This suspension was filtered over a thin layer of Hyflo filter aid.

The precipitates of the two batches were washed with acetone, pooled, and extracted with ether in a Soxhlet apparatus for 2 to 3 days for the purpose of removing the fatty acids. The contents of the thimble were taken up in petroleum ether-methanol (9:1) and filtered. The filtrate was concentrated to a very small volume and the phosphatide was precipitated almost quantitatively with an excess of acetone. The dried precipitate (25.5 gm.) was dialyzed against running water for 24 hours for the purpose of removing inorganic material. The filtration of the dialyzed suspension was difficult in spite of the Hyflo filter aid. It was expedited upon addition of an equal volume of acetone and acidification of the suspension with dilute HCl. The dried precipitate containing traces of cerebrosides was taken up in petroleum ether-methanol (9:1) and run through a column of Al₂O₃ for the selective adsorption of cerebrosides (8). The sphingomyelin was recovered from the concentrated solution by precipitation with acetone (16 gm.). It was recrystallized from 800 cc. of hot ethyl acetate to which 8 cc. of methanol were added. The following results were obtained upon analysis of this substance, which is free of hydrolecithin: 3.28 per cent N, 3.76 per cent P (N:P ratio 2:1).

Properties of Sphingomyelin from Brain—Brain sphingomyelin is a white crystalline substance easily soluble in benzene, soluble in warm alcohol and hot ethyl acetate. It is insoluble in ether and acetone. The substance discolors at 190-195° and melts between 212-213°. [α]D² = +6.25° (4 per cent solution of sphingomyelin in a mixture of chloroform-methanol, 1:1); iodine number, 42.5.

Products of Acid Hydrolysis of Sphingomyelin from Brain. Fatty Acids—
Two portions, each 6 gm., of sphingomyelin (hydrolecithin-free) were refluxed in separate flasks each containing 200 cc. of a solution of 10 per cent sulfuric acid in methanol. After cooling, the methyl esters were extracted several times with petroleum ether. The petroleum ether fractions from both flasks were pooled, washed once with water, and concentrated to dryness. Weight of the methyl esters, 5.2 gm. The methyl esters were taken up in 10 volumes of absolute alcohol (52 cc. for 5.2 gm.) and allowed to stand at room temperature for 3 hours. The resulting precipitate, Fraction I, was filtered and dried; weight 0.65 gm.

The mother liquor was left in the refrigerator overnight. The precipitate, Fraction II, was filtered and dried; weight 1.4 gm. The final mother liquor was concentrated to a very small volume of an oil. A small amount of white solid material which settled out from the oil while drying in the desiccator was separated. The oil, Fraction III, weighed 2.55 gm. The white material, Fraction IIIA, weighed 0.25 gm. The iodine numbers of the three main fractions were 8.1 (Fraction I), 9.05 (Fraction II), 45.5 (Fraction III). These figures indicate the presence of an unsaturated fatty acid, most of which appears in Fraction III.

Main Fraction I yielded two fractions, Fraction IA (solid) distilling from 91.5–106° at 0.001 mm. pressure, and Fraction IB (semisolid) distilling from 90–96° at 0.001 mm. pressure. Fraction IA was converted to the free acid after saponification with 1 cc. of N aqueous sodium hydroxide in 15 cc. of methanol. The acid was recrystallized several times from petroleum ether at room temperature; m.p. 80°; mol. wt. by titration, with 0.1 N sodium ethylate, 372; iodine number 2.56. These figures are in close agreement with the properties of lignoceric acid (m.p. 82°, mol. wt. 368). Fraction IB was converted to the free acid and found to contain some lignoceric acid, which was not further purified.

Main Fraction II yielded two fractions, Fraction IIA (solid) distilling from 70–87° at 0.001 mm. pressure, and Fraction IIB (semisolid) distilling from 69–97° at 0.0075 mm. pressure. Fraction IIA was converted to the free acid as described above and recrystallized several times from a small volume of petroleum ether at room temperature; m.p. 69°; mol. wt. by titration, 282; iodine number 2.61. These results indicate that the fatty acid is stearic acid (m.p. 69°, mol. wt. 284). Fraction IIB, a small part of the main fraction, was a mixture of lignoceric and stearic acids.

Main Fraction III was converted directly to the fatty acids by saponification with 0.1 N alcoholic sodium hydroxide and acidification with dilute hydrochloric acid. The fatty acids were taken up in 40 cc. of petroleum ether.

1 In the saponification of nervonic acid alcoholic sodium hydroxide is used rather than aqueous sodium hydroxide because the nervonic acid is not completely saponified in an aqueous solution.
ether and placed in an insulated container which was kept at $-10^\circ$ by means of dry ice. After 30 minutes the saturated acids had settled. The unsaturated acid in the filtrate was remethylated and distilled at a pressure of 0.001 mm. Two fractions were obtained, Fractions IIIA and IIIB.

Fraction IIIB was converted to the free acid as described above and re-crystallized from 5 cc. of petroleum ether at $-10^\circ$; m.p. 41.5$^\circ$; mol. wt., by titration with 0.1 N sodium ethylate, 367; iodine number 66. Nervonic acid, m.p. 41.5; mol. wt. 366; iodine number 69.

Fraction IIIA contained small amounts of stearic acid and nervonic acid.

The nervonic acid from Fraction IIIB was hydrogenated for 4 hours with palladium charcoal as a catalyst. The hydrogenated substance melted at 81.5$^\circ$. Lignoceric acid, m.p. 82$^\circ$. This melting point substantiates the fact that the unsaturated acid is nervonic acid which was converted by hydrogenation to lignoceric acid.

In another experiment Adams' PtO$_2$ (obtained from the American Platinum Works, Newark, New Jersey) was used as a catalyst in the hydrogenation of nervonic acid but did not prove to be so satisfactory as the palladium charcoal.

The fractionation of the fatty acids from brain yielded lignoceric, stearic, and nervonic acids in the approximate proportions of 1:2:2.

Isolation of Sphingosine Sulfate (Sphingosine-Hydrosphingosine Mixture) —After the removal of the fatty acid esters, the acid hydrolysate of the spingomyelin was neutralized with 4 N alcoholic KOH. The potassium sulfate was filtered off and the filtrate was made just acid to litmus with glacial acetic acid. The solution was made strongly alkaline with potassium hydroxide and the sphingosine was extracted with ether. The ether extract was washed with water and dried over Na$_2$SO$_4$.

The iodine number of this crude base was 41.5. It was recrystallized from petroleum ether at room temperature. The insoluble Fraction A was filtered and the mother liquor was left in the refrigerator overnight. The precipitate (Fraction B) was filtered. The iodine numbers of Fractions A and B were 46 and 19.4 respectively.

0.85 gm. of Fraction A was converted to the sulfate (weight 0.94 gm.). It was recrystallized from 25 cc. of hot absolute alcohol and allowed to stand at room temperature. The precipitate (0.6 gm.) had an iodine number of 46. (The theoretical iodine numbers are 72.9 for sphingosine sulfate, 0 for dihydrosphingosine sulfate.)

$$(C_{19}H_{37}NO_7)_2 \cdot H_2SO_4 \quad \text{and} \quad (C_{19}H_{39}NO_8)_2 \cdot H_2SO_4$$

Calculated, N 4.02

Found, " 3.82

After repeated fractionations of the sphingosine sulfate-dihydrosphingo-
sine sulfate mixture from absolute alcohol, we finally obtained a sphingo-
sine sulfate preparation which had an iodine number of 60.

Preparation of Dihydrosphingosine Sulfate—50 mg. of recrystallized Frac-
tion A (iodine number 60) were dissolved in 50 cc. of glacial acetic acid
and hydrogenated for 4 hours with PtO₂ as a catalyst. The iodine number
of the hydrogenated product was 4.9. Fraction B of the crystallized
sphingosine sulfate (iodine number 19.4) was also hydrogenated under
the same conditions and yielded a substance with an iodine number of 4.4.

We were unable to make the derivatives of sphingosine and dihydrosphin-
gosine described by Carter and his associates (6) because of the lack of
sufficient material. However, the isolation of the two fractions of sphingo-
sine sulfate with respective iodine numbers of 60 and 19.4 and the conver-
sion of both to hydrosphingosine sulfate by hydrogenation justify the
assumption that sphingosine sulfate obtained after hydrolysis of sphingo-
myelin is a mixture of sphingosine sulfate and dihydrosphingosine sulfate.

Preparation of Pure Sphingomyelin from Beef Spleen

The method employed for the isolation of hydrolecithin and the prepara-
tion of pure sphingomyelin from the ether-insoluble phosphatide mixture
from spleen is essentially the same as that previously reported for lung
(4, 9). The yield of sphingomyelin from 50 pounds of spleen was 6.5 gm.

The analysis of the recrystallized substance gave the following results:
3.18 per cent N, 3.77 per cent P (N:P ratio 2:1).

Properties of Sphingomyelin from Spleen—Spleen sphingomyelin is a
white crystalline substance soluble in benzene, warm alcohol, and hot
ethyl acetate, insoluble in ether and acetone. It can be easily recrystal-
lized from a large volume of hot ethyl acetate to which a few cc. of methanol
have been added; m.p. = 217°; [α]₂² = +6.25° (4 per cent solution of sphin-
gomyelin in a mixture of chloroform-methanol, 1:1); iodine number 33.5.

Fatty Acids of Pure Sphingomyelin Prepared from Spleen—4.3 gm. of
sphingomyelin obtained by the procedure as described for lung (4) were
refluxed for 4 hours with 143 cc. of 10 per cent sulfuric acid in methanol.
After cooling, the methyl esters were extracted with three portions of
petroleum ether. The pooled extracts were concentrated to dryness. The
methyl esters weighed 1.7 gm.

On redistillation of the methyl esters a liquid fraction and a solid frac-
tion were obtained. The liquid fraction distilled from 85–96.5° at a pres-
sure of 0.001 mm., while the solid fraction distilled from 90–100.6° at a
pressure of 0.0025 mm.

The liquid fraction was converted to the free acid by saponification with
1 cc. of aqueous sodium hydroxide in 15 cc. of methanol. The acid was
recrystallized from petroleum ether several times at 0°; m.p. 61°; mol. wt.,
by titration with 0.1 N sodium ethylate, 264; iodine number 0. Palmitic
acid, m.p. 62°; mol. wt. 256. It is evident that the fatty acid is palmitic acid.

The solid fraction was converted to the free acid as described above. The acid was recrystallized from petroleum ether several times at room temperature; m.p. 80°; mol. wt. by titration 385; iodine number 0. Lignoceric acid, m.p. 82°; mol. wt. 368.

Sphingosine sulfate was isolated in crystalline form from acid hydrolysis of sphingomyelin as described for brain sphingomyelin. The iodine number of this preparation was 36.7 (iodine number of unsaturated sphingosine 72.9). After hydrogenation with PtO₂ (Adams' catalyst) this substance gave an iodine number of 0.

DISCUSSION

Sphingomyelin of brain is different in regard to its component fatty acids from the sphingomyelin of visceral organs like lung and spleen.² In brain sphingomyelin an unsaturated fatty acid, namely nervonic acid, is found in larger amounts, while its saturated compound, lignoceric acid, is present in smaller quantities than in other organs.

Palmitic acid is not present in sphingomyelin of brain after it is freed of hydrolecithin (dipalmityl lecithin), while in the visceral organs palmitic acid is found together with lignoceric acid as the only fatty acid constituents of sphingomyelin.

Stearic acid is present together with nervonic acid as the main constituent fatty acid of brain sphingomyelin. It has not been found in the sphingomyelin of other organs.

Sphingomyelin of spleen contains palmitic acid and lignoceric acid in equal quantities. Its fatty acid components are thus essentially the same as those of lung sphingomyelin.

The physiological significance of the difference of the component fatty acids of brain sphingomyelin and the sphingomyelin of visceral organs is not known.

The sphingosine fraction of the hydrolysate was isolated from brain as well as from spleen sphingomyelin. The iodine number of the apparently uniformly crystallized sphingosine sulfate of both organs showed that this substance is a mixture of hydrosphingosine and unsaturated sphingosine. The sphingosine groups of the sphingomyelin thus consist of saturated and unsaturated sphingosine, in analogy to the composition of the cerebrosides as previously shown by Carter and his associates (5, 6).

² Brain is an ectodermal organ. Visceral organs like lung and spleen are of endodermal and mesenchymal origin.
SUMMARY

1. A procedure for the preparation of pure brain sphingomyelin free of hydrolecithin is described.

2. The fatty acids present in brain sphingomyelin are different from those present in other organs investigated. The component fatty acids of brain sphingomyelin are stearic, nervonic, and lignoceric acids, while lignoceric acid and palmitic acid are the component fatty acids of sphingomyelin prepared from spleen and lung.

3. Sphingosine isolated after acid hydrolysis of brain as well as spleen sphingomyelin is a mixture of hydrosphingosine and unsaturated sphingosine.

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THE CHEMICAL NATURE OF THE FATTY ACIDS OF BRAIN AND SPLEEN SPHINGOMYELIN. THE OCCURRENCE OF SATURATED AND UNSATURATED SPHINGOSINES IN THE SPHINGOMYELIN MOLECULE

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