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Studies on the Structure of Sphingomyelin. II. Performic and Periodic Acid Oxidation Studies

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A pure sphingomyelin was prepared from beef brain according to the procedure of Thannhauser and Boncoddo. This phosphatide was hydroxylated with performic acid and then oxidatively cleaved with periodate to yield formic acid. Subsequent oxidation by permanganate and hydrolysis with hydrochloric acid gave an amino acid which proved to be identical with serine. This evidence indicates that the phosphorylcholine group of this sphingomyelin is attached to the primary hydroxyl group of sphingosine. The structure is therefore best represented by formula I.

The purpose of this paper is to supply further evidence for the position of attachment of the phosphorylcholine group on the sphingosine component of sphingomyelin. The previous paper[4] demonstrated that phosphorylcholine does indeed exist as a unit in sphingomyelin and is linked to the primary hydroxyl group of sphingosine. The previous paper presents evidence obtained from the treatment of the hydrolyzed products of sphingomyelin, while this paper deals with oxidation studies on the intact sphingomyelin molecule.

The sphingomyelin used in these experiments was prepared from beef brain according to the procedure of Thannhauser and Boncoddo[5] and the product obtained was subjected to further purification. Cerebrosides were removed by fractional crystallization from ethyl acetate–methyl alcohol, and pyridine, and by the use of Al_2O_3 adsorption columns. Glycerophosphatides were eliminated by selective hydrolysis with alkali at 37°, and non-phosphorylcholine phospholipids such as cephalin were further removed by the use of MgO according to the procedure of Taurog and co-workers.[6] The separation of inorganic ions was accomplished by dialysis against running water. Finally the sphingomyelin was recrystallized several times from an ethyl acetate–methyl alcohol mixture to give a white crystalline product melting at 170–171° with very slight decomposition. The sphingomyelin is insoluble in ethyl ether and acetone and soluble in formic acid, acetic acid, petroleum ether, mixtures of petroleum ether with chloroform or alcohol and in hot ethyl acetate–methanol. This phospholipid showed no signs of discoloration or decomposition after standing at room temperature in a desiccator for one year.

The following tests and analyses indicate the purity of the sphingomyelin: 1. Nitrogen and phosphorus analyses gave the expected theoretical N:P ratio of 2:1 [N, 2.80 (Kjeldahl); P, 3.01].

2. Carbon and hydrogen analyses[7] were as follows: C, 65.35; H, 10.96. (These are in agreement with though not *conclusive* for a sphingomyelin containing stearic acid.) Calculated values for "stearyl" sphingomyelin are C, 65.71; H, 11.44. The fatty acid was not isolated and identified since the nature of this acid was not critical in the investigation.

3. A sample of the sphingomyelin was hydrolyzed with 6 *N* HCl for 15 minutes, cooled and filtered. Subsequent paper chromatographic analysis of the filtrate failed to show a spot due to sugar, whereas a pure cerebroside treated in the identical manner gave a pronounced spot due to sugar.

A Molisch test on the above filtrate was also essentially negative. This test carried out on a known solution of sugar and on a pure cerebroside gave strong positive tests. The results demonstrate the absence of carbohydrate impurities.

4. Solid film infrared absorption spectra of the sphingomyelin[8] gave all the absorption peaks expected for this molecule. Thus the typical amide peaks appeared at 6.09 and 6.45 μ; possible *trans* double bond peak and covalent phosphate peak at 10.30 μ; NH stretching and hydroxyl group peak with possible hydrogen bonding and shifting at 3.10 μ; and a long chain hydrocarbon peak at 13.85 μ. It is noteworthy that the absence of the ester carbonyl and carboxyl carbonyl bands at 5.7–5.9 μ is confirmatory evidence that the sphingomyelin was essentially free from cephalin, lecithin, glycerides and serine phospholipids. Further work is being done on the infrared spectra of sphingomyelin and other phosphatides.

Since the sphingomyelin preparation satisfied all the criteria of purity which were employed, the reactions formulated below were performed in order to determine the point of attachment of the phosphorylcholine group. The schemes illustrate the different products that might be expected from the two possible structures of sphingomyelin.

Since Swern and co-workers[9] had shown that higher unsaturated fatty acids can be hydroxylated in very good yield with performic acid, and since sphingosine possesses a long unsaturated hydrocarbon chain, it appeared that this procedure should be applicable for the hydroxylation of sphingomyelin. Hence the sphingomyelin (I, II) was treated with performic acid using a modified procedure of Swern and co-workers and hydrolyzed with alkali to obtain the hydroxylated product. A portion of the

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(4) G. Rouser, J. F. Berry, G. Marinetti and E. Stotz, THIS JOURNAL, **75**, 310 (1953).

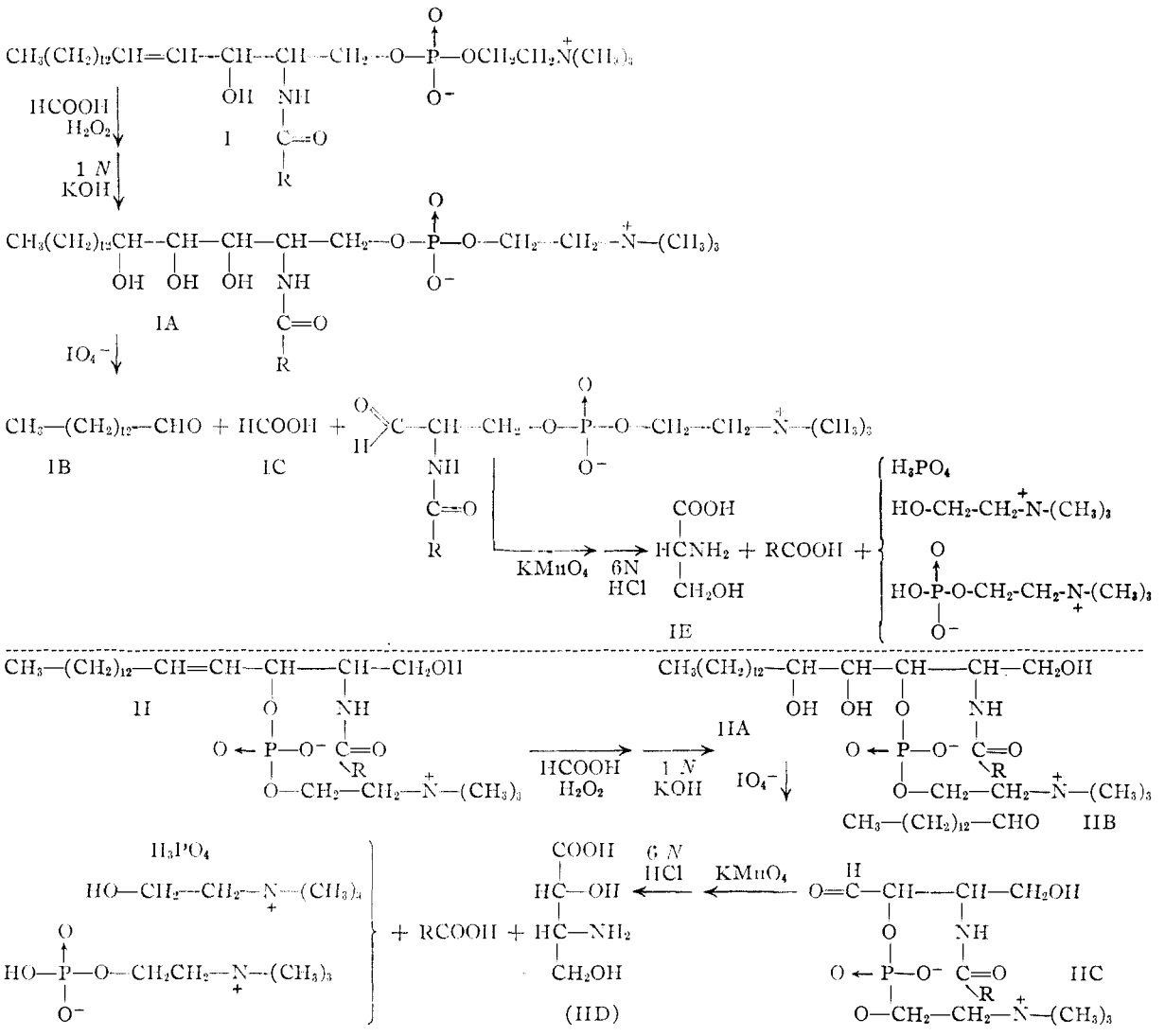
(5) S. J. Thannhauser and N. F. Boncoddo, *J. Biol. Chem.*, **172**, 135 (1948); **172**, 141 (1948).

(6) A. Taurog, C. Entenman, B. A. Fries and I. L. Chaikoff, *ibid.*, **155**, 19 (1944).

(7) Analyses by Miss C. King of the Micro-Tech Laboratories.

(8) The authors wish to thank Dr. R. Fowler and Mr. A. Durbetaki of the Physiology Dept. of this institution for determining the infrared spectra of sphingomyelin and its hydroxylated derivative.

(9) D. Swern, G. Billen, T. Findley and J. Scanlan, THIS JOURNAL, **67** 1786 (1945).



hydroxylated sphingomyelin (IA, IIA) was isolated and found to be more soluble in methanol than the original sphingomyelin. The infrared spectrum of the hydroxylated product revealed a stronger absorption band with shifting to 3.01 μ in the hydroxyl region than did sphingomyelin.

The hydroxylated product (IA, IIA) was freed of excess formic acid by repeated vacuum distillation until the distillate was free of titratable acid. The residue in the flask was oxidized with periodate using a modified procedure of Huber[10] who utilized periodic acid for oxidative degradation of dihydroxystearic acid. After oxidation of the sphingomyelin the *p*H of the reaction mixture fell and the suspension gave a strong positive test for aldehyde with Tollens reagent, compatible with formic acid liberation and aldehyde formation.

Distillation of the mixture *in vacuo* using a long column and efficient trap and titration of the water-clear distillate with 0.01 *N* NaOH, yielded an 88% recovery of the formic acid. Additional evidence that the distillate contained formic acid was the fact that it gave a positive Tollens test.

The remaining aqueous residue in the distilling flask was extracted with ether to give approximately 85% of the theoretical amount of myristaldehyde (IB, IIB). Treatment of an alcoholic solution of this compound with 2,4-dinitrophenylhydrazine gave positive evidence for the presence of the carbonyl group. The 2,4-dinitrophenylhydrazone was isolated and recrystallized from alcohol, m.p. 101–103°. The 2,4-dinitrophenylhydrazone of an authentic sample of myristaldehyde melted at 102–103°.

The aqueous residue remaining after ether extraction was treated with ethylene glycol to destroy the excess periodate and then oxidized with permanganate under mild conditions. After hydrolysis with HCl a compound (IE, IID) was obtained which gave a strong positive ninhydrin test. When this compound was subjected to paper chromatography, it was found to move identically with serine. The finding of this amino acid lends confirmation to the structure of sphingosine as proposed by Carter and co-workers.[11]

A portion of the original sphingomyelin was also treated in the same manner as described above except for the omission of the performic acid hydroxylation. This treatment failed to yield formic acid or serine.

(10) F. Huber, THIS JOURNAL, **73**, 2730 (1951).

(11) H. E. Carter, F. S. Glick, W. P. Norris and G. E. Phillips, *J. Biol. Chem.* **170**, 285 (1947).

The purpose of this procedure was to rule out the possibility that serine or formic acid could have risen by another pathway or from impurities such as carbohydrate or serine phospholipids.

In order to eliminate the possibility that formic acid or KOH treatment caused hydrolysis of the sphingomyelin prior to periodate oxidation, separate samples of the compound were individually subjected to hydrolysis with 1 *N* KOH, 96% HCOOH and 1 *N* HCl at 37° for 24 and 48 hours. At the end of each time interval samples were analyzed by paper chromatography for choline and phosphorylcholine and found to be negative. This was not unexpected since sphingomyelin has been shown by Schmidt and co-workers[12] to be stable toward NaOH hydrolysis at 37°. In fact the sphingomyelin used in this experiment was purified by treatment with alkali at 37° as mentioned previously.

The question also arises whether periodic acid cleaves the amide link on the sphingosine molecule, or the ester link between sphingosine and phosphorylcholine. Carter and co-workers[11] have shown that dihydrosphingosine amides, *i.e.*, N-benzoyl- and N-acetyldihydrosphingosine, are not cleaved by periodic acid. Fleury and Paris[13] and Burmaster[14] have reported that periodic acid does not attack the ester link in either α- or β-glycerophosphoric acid. By analogy, periodic acid would not be expected to cleave the phosphate ester link in sphingomyelin which has been shown to be more stable toward hydrolysis than the corresponding phosphate ester link of glycerophosphatides. The above data therefore give reassuring evidence that the conditions of the experiment should not chemically alter the sphingomyelin in such a manner as to give misleading results.

Evidence from the above experiments demonstrates conclusively that the structure of the sphingomyelin prepared is best illustrated by I.[15]

Experimental

Hydroxylation of the Sphingomyelin.—Four hundred mg. (approximately 0.5 mmole based on the average molecular weight of stearyl lignoceryl sphingomyelin) was dissolved in 10 ml. of redistilled 96% formic acid and 0.4 ml. of concentrated hydrogen peroxide (3.5 mmole) was added. The resulting clear solution was placed in the dark at 37° for 12 hours, then at 5° for 12 hours. The excess formic acid and peroxide were removed *in vacuo* at 40°. To the residue was added 15 ml. of 1 *N* KOH and the resulting suspension shaken continuously in the dark at 37° for 22 hours. Neutralization was carried out by the addition of the appropriate amount of H_2SO_4 (approximately 1.5 ml. of 10 *N* H_2SO_4). A slight excess of the acid was added to bring the suspension to *p*H 1.3. In a similar experiment a portion of the hydroxylated product (IA, IIA) was isolated from such a mixture and purified for subsequent infrared analysis. In order to remove any formic acid liberated on hydrolysis, the suspension was distilled repeatedly *in vacuo* at 40° until the distillate was free of titratable acid.

Oxidation with Periodate.—The residue remaining from vacuum distillation was brought to *p*H 5.4 with Na_2HPO_4 and diluted to 50 ml. with distilled water. To the suspension was added 15 ml. of 0.2 *M* $NaIO_4$ (3 mmole) and the mixture shaken continuously in the dark for 12 hours at 37°. The resulting suspension gave a strong positive test for the carbonyl group with Tollens reagent. The *p*H of the solution fell to 4.3. Extraction was then carried out three times with ethyl ether in order to remove the compound which was split off. The ether extracts were dried over Na_2SO_4, filtered and the ether evaporated off to yield approximately 85 mg. of a white, waxy, low melting solid (IB, IIB) (theoretical 101 mg.).

To prepare a derivative of this low melting compound, the following procedure was used. Two ml. of concentrated H_2SO_4 was added to 0.4 g. of 2,4-dinitrophenylhydrazine, after which 3 ml. of water was added dropwise with swirling until solution was complete. To this warm solution was added 10 ml. of 95% ethanol. The compound (IB, IIB) was dissolved in 10 ml. of ethanol and the appropriate amount of the freshly prepared 2,4-dinitrophenylhydrazine solution was then added. The hydrazone formed almost immediately. A portion of the derivative was recrystallized from ethanol as a fine yellow crystalline precipitate, m.p. 101–103°. The 2,4-dinitrophenylhydrazone prepared from an authentic sample of myristaldehyde and recrystallized from alcohol, melted at 102–103°.

Isolation and Titration of the Liberated Formic Acid.—The aqueous phase was treated with 3 ml. of 85% H_3PO_4 to give *p*H 1.8. A white flocculent material formed. An efficient trap and long distilling column were set up and the liberated formic acid (IC) distilled *in vacuo* at 40°. The formic acid was collected in a flask immersed in an ice-salt-bath. The long column was used to prevent any possibility of splashing acid into the collecting flask. The distillate containing the formic acid was titrated with 0.01 *N* NaOH to *p*H 6.7 using chlor phenol red indicator. This required 43.5 ml. of base (theoretical 50 ml.). This acid also gave a positive Tollens test.

Oxidation with Potassium Permanganate and HCl Hydrolysis.—The residue in the flask was diluted to 50 ml. with water. The resulting suspension was treated with ethylene glycol to destroy the excess periodate. The mixture was subsequently oxidized with excess potassium permanganate at 40° for 15 min. The excess permanganate and brown precipitate which formed were decolorized with $NaHSO_3$ and the solution brought to a concentration of 6 *N* with concentrated HCl and refluxed for 3 hours. The unhydrolyzed material (fatty acid) was filtered off and the filtrate distilled *in vacuo* to remove most of the excess HCl.

Identification of Serine.—The solution remaining after distillation was brought to *p*H 7.4 with NaOH and a slight excess of $BaCl_2$ was added to precipitate the phosphate, iodate and sulfate ions. The precipitate which formed was removed by centrifugation and the supernatant adjusted to *p*H 7.4. This solution gave a strong ninhydrin color reaction (blue-violet color). The bulk of this solution was evaporated to dryness and the residue extracted with ethanolic hydrochloric acid (0.5 *N* HCl in 85% ethyl alcohol). Chromatograms were run on the alcohol extracts in the following solvent systems: phenol saturated with water, collidine saturated with water and *n*-butanol saturated with water. The spots were developed by dipping in 0.2% ninhydrin in acetone and heating at 100° for 10 minutes. The unknown compound (IE, IID) gave a spot in all these solvent mixtures which moved identically with an authentic sample of pure serine, and was thus proved to be serine (IE).

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(12) G. Schmidt, J. Benotti, B. Hershman and S. J. Thannhauser, *J. Biol. Chem.*, **166**, 505 (1946).

(13) P. Fleury and R. Paris, *Compt. rend.*, **196**, 1416 (1933).

(14) C. F. Burmaster, *J. Biol. Chem.*, **164**, 233 (1946).

(15) After the completion of this work a paper by Dr. Kurt Mislow appeared[16] which indicates by means of infrared spectra that the double bond in both sphingosine and cerebron has the *trans* form. Our studies likewise indicate that the double bond in sphingomyelin has the *trans* configuration since the 10.30 μ band in the infrared spectrum of the sphingolipid is significantly diminished upon hydroxylation.

(16) Kurt Mislow, THIS JOURNAL, **74**, 5155 (1952).