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Studies on the Structure of Sphingomyelin. III. Quantitative Data on the Peroxide and Periodate Uptake during the Oxidation of Sphingomyelin

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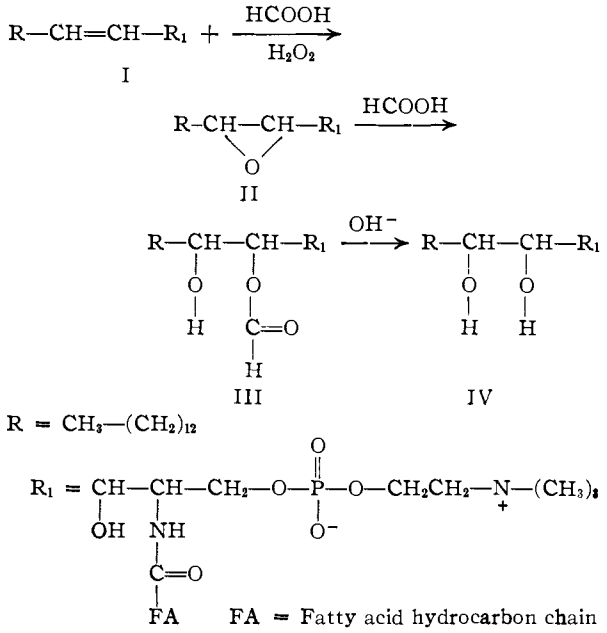
Sphingomyelin was hydroxylated with performic acid and subsequently oxidatively cleaved with periodic acid. Quantitative measurements showed that one mole of peroxide and two moles of periodate were consumed for each mole of sphingomyelin which was oxidized. The postulated intermediates during the hydroxylation of sphingomyelin were substantiated by means of infrared spectroscopy.

In previous experiments the authors[2a,b] established the position of attachment of the phosphorylcholine group on the sphingosine moiety of sphingomyelin. One method which was used[2b] consisted in hydroxylating sphingomyelin with performic acid and subsequently cleaving the product oxidatively with periodic acid. The present paper gives quantitative data regarding the peroxide uptake during performic acid oxidation and the periodate consumption during oxidative cleavage. The data are in excellent agreement with the expected theoretical results which should be obtained if the phosphorylcholine group is attached to the primary hydroxyl group of sphingosine. Thus one mole of peroxide and two moles of periodate were consumed for each mole of sphingomyelin which was oxidized.

Furthermore, the reaction mechanism proposed for the performic acid oxidation of a double bond was substantiated by means of infrared analysis of the reaction products. The proposed reaction is given below.[3] According to the mechanism the



double bond in structure I (sphingomyelin) is first oxidized by performic acid to an epoxide II which in the presence of excess formic acid is rapidly converted to the hydroxyformoxy derivative III (hydroxyformoxysphingomyelin). This latter compound is easily converted to the hydroxylated derivative IV (hydroxylated sphingomyelin) by the action of base. Under the conditions used in the experiment, it was not possible to isolate the epoxide intermediate II. However, the products III and IV were isolated and their structures confirmed by means of infrared analysis. The original sphingomyelin I did not contain an ester carbonyl band in the region 5.7–5.9 μ, but after performic acid oxidation the isolated hydroxyformoxysphingomyelin III was shown to have a strong band at 5.81 μ in the ester carbonyl region. It also was noted that the amide bands in compound III were displaced in comparison to the original sphingomyelin I (in which the typical amide bands occurred at 6.09 and 6.54 μ) such that a triplet resulted with the greatest absorption at 6.25 μ but with less intense bands at 6.06 and 6.46 μ. This may possibly be due to interaction of the amide linkage with the neighboring formoxy group. After hydrolysis of the hydroxyformoxysphingomyelin III an equivalent amount of formic acid was liberated, the ester carbonyl band at 5.81 μ completely disappeared, and the amide bands returned to their normal positions. Furthermore, the hydroxylated sphingomyelin IV showed a greater absorption in the hydroxyl region near 3.0 μ than the original sphingomyelin, with slight shifting of the band toward a shorter wave length (from 3.10 to 3.01–3.05 μ). This also was observed in the spectrum of compound III. It is reasonable to assume that the greater absorption in the hydroxyl region and the displacement of the band toward shorter wave length are due to the fact that compound III has two hydroxyl groups and compound IV has three hydroxyl groups, whereas the original sphingomyelin I has only one such functional linkage. It is further recognized that the absorption band near 3.0 μ is a summation of absorption due to both the OH and the NH linkages.

In addition to the above findings in the infrared spectra of sphingomyelin and its derivatives, it was further noted that the band near 10.3 μ was significantly diminished and altered after hydroxylation.[2b] Since the *trans* double bond (but not the *cis*) is associated with a band in this region,[4] the finding indicated that the double bond in sphingomyelin has the *trans* configuration. Further investigations confirmed this hypothesis and will be presented in a succeeding paper.[5]

(1) National Science Foundation Fellow.

(2) (a) G. Rouser, J. F. Berry, G. Marinetti and E. Stotz, THIS JOURNAL, **75**, 310 (1953); (b) G. Marinetti, J. F. Berry, G. Rouser and E. Stotz, *ibid.*, **75**, 313 (1953).

(3) D. Swern, G. Billen, T. Findley and J. Scanlan, *ibid.*, **67**, 1786 (1945).

(4) F. A. Miller, "Organic Chemistry," Vol. III, Edited by H. Gilman, John Wiley and Sons, Inc., New York, N. Y., 1953, p. 154.

(5) G. Marinetti and E. Stotz, THIS JOURNAL, **76**, 1347 (1954).

Experimental

Peroxide Uptake During the Oxidation of Sphingomyelin I with Performic Acid.—The preparation and purity of the sphingomyelin used in these experiments have been described in a previous communication.[2b]

Eight hundred milligrams (1.0 mmole) of sphingomyelin was dissolved in 30 ml. of 90% formic acid with slight warming, exactly 1 ml. of 2 *M* H_2O_2 (2 mmoles) added, and the solution brought to 50 ml. in a volumetric flask by the addition of 90% formic acid. A blank was prepared by adding exactly 1 ml. of 2 *M* H_2O_2 solution to another 50-ml. volumetric flask and diluting to 50 ml. with 90% formic acid. Immediately 1-ml. aliquots were taken out and analyzed for H_2O_2.

The flasks were immersed in a water-bath at 40° for 6 hr. At the end of 4- and 6-hr. intervals additional 1-ml. aliquots were removed for H_2O_2 analysis. The aliquots were analyzed as follows: to the 1-ml. aliquots in 50-ml. erlenmeyer flasks were added 5 ml. of water, 1 ml. of 1 *N* HCl, 1 drop of 1% ammonium molybdate solution and 3 ml. of freshly prepared 10% KI solution. The flasks were tightly stoppered, placed in the dark for 5 minutes, and then titrated to the starch end-point with standard 0.01 *N* $Na_2S_2O_3$. It was found that after 4 hr. the reaction was complete and that 1.12 mmoles of H_2O_2 were consumed for each mmole of sphingomyelin. According to Swern[3] 1.025 mmoles of H_2O_2 is required to oxidize 1 mmole of double bond instead of the theoretical 1.0 mmole.

Isolation of the Hydroxyformoxysphingomyelin III.—The solution remaining after the performic acid oxidation was placed in a beaker and concentrated to dryness with a stream of nitrogen at room temperature. The white residue was washed by suspending three times in cold acetone and the precipitate removed by centrifugation and dried *in vacuo* in a Fisher pistol at 56° for 3 hours over NaOH to yield 580 mg. of dry white powder, m.p. 194–198° (with decomposition) which was completely free of acid. The acetone contained additional amounts of product, but this was discarded. Both the original sphingomyelin and the hydroxyformoxy derivative were analyzed in the infrared region of the spectrum as Nujol mulls.[6] The major bands in compound I occurred at 3.10, 6.09, 6.45, 8.12, 9.18, 9.44, 10.32 and 13.86 μ and those for compound III at 3.01, 5.81, 6.06, 6.25, 6.46, 8.12, 9.18, 9.44, 10.28 and 13.86 μ.

Hydrolysis of the Hydroxyformoxysphingomyelin III and Titration of the Liberated Formic Acid.—Two hundred milligrams (0.25 mmole) of the hydroxyformoxysphingomyelin III was hydrolyzed by suspending it in 10 ml. of 1 *N* KOH for 22 hr. at 37°. To the suspension was added a slight excess of 85% H_3PO_4 and 20 ml. of redistilled acetone. The mixture was chilled on ice for 0.5 hr. and centrifuged in the cold. The supernatant was carefully decanted off and subjected to vacuum distillation at 40° in order to obtain the liberated formic acid which was collected in an ice trap. The distillate thus obtained was titrated with standard 0.01 *N* NaOH to the chlor phenol red end-point, requiring 23.8 ml. of base solution which is in good agreement with the theoretical value of 25 ml.

Isolation of the Hydroxylated Sphingomyelin IV and Measurement of the Periodate Uptake During Its Oxidation.—The precipitate obtained upon acetone treatment of the hydrolyzed hydroxyformoxysphingomyelin III was suspended twice in cold acetone and separated by centrifugation. The residue was dried *in vacuo* at 56° for 3 hours and extracted twice with 10-ml. portions of chloroform. The chloroform extracts were evaporated to dryness *in vacuo* to yield 118 mg. of white powder, m.p. 211–212° with decomposition (hydroxylated sphingomyelin IV), which was dried thoroughly over NaOH and P_2O_5 at 56° *in vacuo*.

One hundred milligrams (0.126 mmole) was placed in a 50-ml. volumetric flask and 15 ml. of methanol added. To the mixture was added 5 ml. of 0.1 *N* $NaIO_4$ (aqueous) and methanol added to make a total volume of 50 ml. A blank solution was prepared by adding 5 ml. of 0.1 *N* $NaIO_4$ solution to another 50-ml. volumetric flask and diluting to the 50-ml. mark with methanol.

The flasks were tightly stoppered and placed in a water-bath at 37° for 5 hr. with constant shaking. Fine white needles began to form in the flask containing the hydroxylated sphingomyelin, whereas the blank solution remained perfectly clear. The reaction was allowed to proceed for 11 hr. more. Upon centrifugation of the contents of the flasks, the solution containing the hydroxylated sphingomyelin yielded a fine white crystalline precipitate, whereas the blank did not yield any residue. The white crystalline precipitate was believed to be $NaIO_3$ which formed as a result of the oxidation. This was confirmed by dissolving it in 3 ml. of water, adding 1 drop of concd. HNO_3 and then adding 2 drops of 3% $AgNO_3$ solution.[7] The expected insoluble precipitate of $AgIO_3$ formed immediately. When the blank solution and pure crystals of $NaIO_4$ were treated in the identical manner, completely negative results were obtained. Further indication that oxidation of the sphingomyelin had occurred was demonstrated by the fact that the sphingomyelin solution gave a strong positive test with Schiff reagent, whereas the blank solution was entirely negative.

The supernatant solutions obtained after centrifugation were analyzed for periodate by taking 2-ml. aliquots, adding 10 ml. of water, slight excess of solid $NaHCO_3$ and 3 ml. of 10% KI solution. The 50-ml. erlenmeyer flasks containing these solutions were tightly stoppered and let stand in the dark for 3 minutes. The liberated iodine was titrated to the starch end-point with standard 0.01 *N* Na_3AsO_3 solution.

It was found that 0.288 mmole of periodate (theoretical value is 0.252 mmole) was taken up for each 0.126 mmole of hydroxylated sphingomyelin (2:1 ratio) which is in satisfactory agreement with the proposed mechanism.[2b]

Five mg. of the hydroxylated sphingomyelin IV was analyzed in the infrared as a Nujol mull.[6] The major bands occurred at 3.01–3.05, 6.12, 6.42, 8.15, 9.18, 9.40, 10.28 and 13.86 μ.

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(6) Spectra run by Mr. Carl Whiteman of the Chemistry Dept. of this University, using a Perkin-Elmer model 12 AB recording spectrometer, with a sodium chloride prism. The Nujol was pipetted from a hypodermic needle to give drops weighing approximately 6 mg. Five milligrams of sample was mulled in one drop of Nujol.

(7) G. F. Smith, "Analytical Application of Periodic Acid and Iodic Acid and Their Salts," 5th Ed., G. Frederick Smith Chem. Co., Columbus, Ohio, 1950, p. 86.