[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE AND DENTISTRY]

Studies on the Structure of Sphingomyelin. I. Oxidation of Products of Partial Hydrolysis

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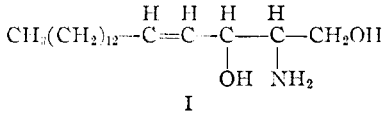
Evidence for the structure of sphingomyelin is presented. Phosphorylcholine has been demonstrated to be a hydrolytic product of sphingomyelin and thus phosphorus and choline are linked together in the intact molecule. After partial alkaline hydrolysis the sphingosine phosphate and sphingosinephosphorylcholine produced are subjected to periodate oxidation. Glycolaldehyde phosphate was demonstrated to be one of the products of the oxidation of sphingosine phosphate and the production of a glycolaldehyde derivative from sphingosinephosphorylcholine was also indicated. This evidence indicates that in the sphingomyelin molecule phosphorylcholine is linked through the primary hydroxyl group on the first carbon atom of sphingosine. The secondary hydroxyl group on the third carbon atom is free.

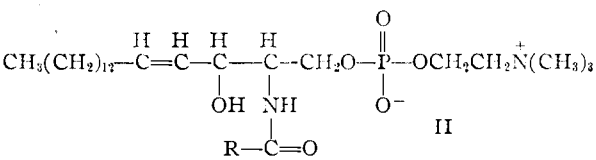
Sphingomyelin was discovered in 1901 by Thudichum,[4] who identified the hydrolytic products as phosphoric acid, fatty acid, choline and a second base of unknown structure now known as sphingosine. The structure of sphingosine has been investigated by Levene and Jacobs,[5] Klenk,[6] Klenk and Diebold,[7] Carter, *et al.*,[8-10] and Ono.[11] Carter and co-workers have established the positions of the amino groups and the two hydroxyl groups of sphingosine and proposed the structure



I

Carter and collaborators[12] isolated from brain and spinal cord the compound dihydrosphingosine which was shown by Thannhauser and Boncoddo[13] to be present together with sphingosine in sphingomyelin.

The manner in which these known hydrolytic products are combined in the intact molecule has also been studied. Levene[14] isolated from a partial hydrolysate of sphingomyelin the compound lignocerylsphingosine, composed of sphingosine and lignoceric acid joined through an amide linkage. The isolation of this compound has recently been confirmed by Thannhauser and Reichel.[15] Since the fatty acid is present in an amide linkage, choline and phosphoric acid must be linked to one or both hydroxyl groups. By analogy to the lecithin molecule, the structure has frequently been assumed to be



II

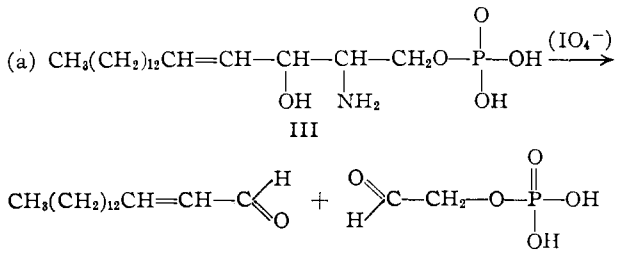
Other authors have assumed that phosphoric acid and choline are linked through the secondary hydroxyl on the third carbon atom of sphingosine. The possibility that phosphoric acid and choline were linked to separate hydroxyl groups has seldom been considered even though there is no direct evidence to the contrary. The assumption that phosphorus and choline are linked together has been made on the basis of analogy and because complete release of phosphate upon acid or alkaline hydrolysis is difficult to obtain, which is consonant with the fact that phosphorylcholine is very difficult to hydrolyze.[16] The possibility that sphingosine phosphate is the compound that is difficult to hydrolyze has not been considered. By analogy to the structure of glycerophosphate, ethanolamine phosphate and serine phosphate,[17-19] all of which are relatively stable to hydrolysis, it can be assumed that sphingosine phosphate is indeed rather stable. Rennkamp[20] reported the isolation of sphingosinephosphorylcholine and phosphorylcholine from acid hydrolysates of sphingomyelin. He used a quantitative determination of the nitrogen and phosphorus of the reinecke salt of the two compounds for identification, but a number of workers (see ref. 21) have found reinecke salt precipitates variable in composition. Booth[22] and King and Small[23] reported the isolation from animal organs of a compound believed to be sphingosinephosphorylcholine, but gave no evidence for the structure of the compound. Fujino[24] has studied the structure of sphingomyelin by a methylation procedure in which he assumes that phosphorus and choline are

(1) Atomic Energy Commission Fellow in the Biological Sciences.
(2) Aided by a grant from the Nutrition Foundation, Inc.
(3) Aided by a grant from the National Foundation for Infantile Paralysis.
(4) J. L. W. Thudichum, "Die chemische Constitution des Gehirns des Menschen und der Tiere," Franz Pietzcker, Tübingen, 1901.
(5) P. A. Levene and W. A. Jacobs, *J. Biol. Chem.*, **11**, 547 (1912).
(6) E. Klenk, *Z. physiol. Chem.*, **185**, 169 (1929).
(7) E. Klenk and W. Diebold, *ibid.*, **198**, 25 (1931).
(8) H. E. Carter, F. J. Glick, W. P. Norris and G. E. Phillips, *J. Biol. Chem.*, **142**, 449 (1942).
(9) H. E. Carter, F. J. Glick, W. P. Norris and G. E. Phillips, *ibid.*, **170**, 285 (1947).
(10) H. E. Carter, F. L. Greenwood and C. G. Hunniston, *Federation Proc.*, **9**, 159 (1950).
(11) K. Ono, *J. Japan. Biochem. Soc.*, **20**, 32 (1948); *C. A.*, **43**, 6267d (1949).
(12) H. E. Carter, W. P. Norris, F. S. Glick, G. E. Phillips and R. Harris, *J. Biol. Chem.*, **170**, 269 (1947).
(13) S. J. Thannhauser and N. F. Boncoddo, *ibid.*, **172**, 141 (1948).
(14) P. A. Levene, *ibid.*, **24**, 69 (1916).
(15) S. J. Thannhauser and M. Reichel, *ibid.*, **135**, 1 (1940).
(16) E. Baer and C. S. McArthur, *ibid.*, **154**, 451 (1944).
(17) E. Baer and M. Kates, *ibid.*, **175**, 79 (1948).
(18) R. H. A. Plimmer and W. J. N. Burch, *Biochem. J.*, **31**, 398 (1937).
(19) R. H. A. Plimmer, *ibid.*, **35**, 461 (1941).
(20) F. Rennkamp, *Z. physiol. Chem.*, **284**, 215 (1949).
(21) H. Wittcoff, "The Phosphatides," Reinhold Publishing Corp., New York, N. Y., 1951, p. 171.
(22) F. J. Booth, *Biochem. J.*, **29**, 2071 (1935).
(23) E. J. King and C. W. Small, *ibid.*, **33**, 1135 (1939).
(24) Y. Fujino, *J. Biochem.*, **39**, 45 (1952).

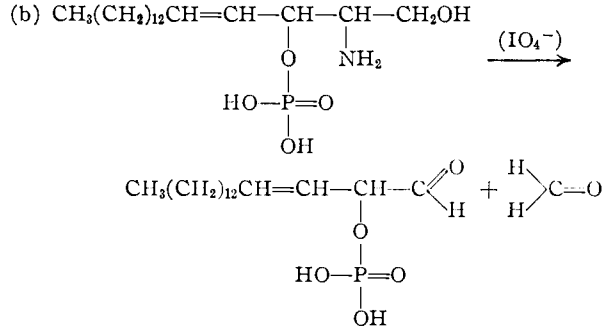
linked together through one or the other hydroxyl group in the sphingosine molecule. After methylation Fujino hydrolyzed the molecule to obtain O-methylsphingosine which he subjected to lead tetraacetate oxidation. The product of the oxidation gave a negative Schiff test and was assumed to be a nitrile because ammonia was liberated on acid hydrolysis. The silver salt of the resulting methoxy fatty acid was identified on the basis of silver and methoxy content. This work is in agreement with that of Nakayama[25] who studied the structure of the cerebroside molecule by the same technique. The lead tetraacetate oxidation of sphingosine may well yield a nitrile but the complete characterization of the product would appear necessary since there are no previous reports in the literature on the oxidation of hydroxyamino compounds of this type by this reagent.

The improved method devised by Thannhauser and Boncoddo[13] was employed by one of us (G.M.) to prepare the sphingomyelin used throughout the present investigation. The properties of the preparation, reported in the succeeding paper, conform to those described by Thannhauser. The first step in structure proof was the direct demonstration of phosphorylcholine as a hydrolytic product of sphingomyelin. This was done by hydrolyzing the preparation with methanolic hydrochloric acid followed by extraction of phosphorylcholine into an aqueous phase with subsequent identification by paper chromatography. This compound can readily be identified by a comparison of the movement on paper with that of an authentic sample of phosphorylcholine,[26] and by elution from chromatograms and identification of phosphoric acid and choline after prolonged alkaline hydrolysis. Choline and orthophosphate were also identified by paper chromatography.

For the determination of the point of attachment of the phosphorylcholine moiety to sphingosine a method based on partial alkaline hydrolysis and periodate oxidation of the sphingosine phosphoric acid esters was used. Since periodic acid oxidation of sphingosine has been conducted by Carter and co-workers[9] and the products have been conclusively identified, the results of periodate oxidation of a sphingosine derivative can be predicted. Sphingosine phosphate and sphingosinephosphorylcholine are possible products of the partial hydrolysis of sphingomyelin. The action of periodic acid on sphingosine phosphate (both possible structures) is shown below. Sphingosinephosphorylcholine would react similarly.



III



The presence of sphingosine phosphate and sphingosinephosphorylcholine in the partial alkaline hydrolysates of sphingomyelin was determined by solvent extraction and paper chromatography. While it would have been possible to conduct the periodate oxidation directly on the mixture of hydrolysis products, the authors chose to perform separate periodate oxidations on solvent fractions shown to contain sphingosine phosphate (petroleum ether) and sphingosinephosphorylcholine (chloroform). The presence of glycolaldehyde phosphate and glycolaldehydephosphorylcholine was demonstrated by a specific color test for glycolaldehyde, and in the case of glycolaldehyde phosphate, by comparison on paper chromatograms with an authentic sample of glycolaldehyde phosphate. The production of glycolaldehyde phosphate by periodate oxidation of sphingosine phosphate lends support to the earlier assumptions and substantiates the work of Fujino.[24] The structure of the sample of sphingomyelin studied was therefore that shown in Fig. 2.

Experimental

Acid Hydrolysis.—Twenty-five mg. of sphingomyelin in 1 ml. of 5% methanolic hydrochloric acid (prepared from absolute methanol and dry hydrogen chloride) was hydrolyzed in a sealed tube at 100° for periods varying from 0.5 to 1.5 hours. The methanol solution was evaporated to dryness and the water-soluble fraction was used for the demonstration of phosphorylcholine.

Basic Hydrolysis.—Basic hydrolysis was performed on 25 mg. of sphingomyelin in 1 ml. of 1 *N* potassium hydroxide in methanol in a sealed tube at 100° for one hour. The products, after solvent separation, were used for periodate oxidation.

Solvent Extraction of Basic Hydrolysates.—The solid that precipitated upon cooling of the basic hydrolysate was removed and dissolved in chloroform. This material was a mixture of unhydrolyzed sphingomyelin and some sphingosinephosphorylcholine. The methanol solution was then adjusted to a *p*H near 7. When the solution is adjusted so that it is slightly acid, a water-soluble solid precipitates and is removed. This material is largely precipitated salts including those of phosphoric acid and some phosphorylcholine. If the solution is adjusted to a *p*H near 8 the methanol contains almost exclusively sphingosinephosphorylcholine. The methanol solution after adjustment to a slightly acid *p*H was evaporated to dryness and extracted at room temperature successively with 1.5 ml. of petroleum ether, chloroform and methanol. For chromatographic purposes 0.1 cc. of each fraction was applied to the paper in as small a spot as possible.

Treatment of Paper for Chromatography.—Whatman No. 1 filter paper washed with 2 *N* acetic acid followed by 50 and 95% aqueous ethanol was used throughout. After chromatography in phenol saturated with water, chromatograms must be allowed to dry for 48 hours or immediately washed twice with xylene and once with ether. Other solvents were allowed to evaporate until the paper was dry.

Identification of Phosphorylcholine, Choline and Orthophosphate.—Phosphorylcholine was identified by paper

(25) T. Nakayama, *J. Biochem.*, **37**, 309 (1950).

(26) We are indebted to Dr. Richard F. Riley of the University of California at Los Angeles for a sample of synthetic phosphorylcholine.

chromatography in phenol saturated with water (R_f 0.15) and the *t*-butanol–picric acid–water solvent of Hanes and Isherwood[27] (R_f 0.15). The compound was located on chromatograms by spraying with the modified Hanes and Isherwood phosphate reagent described by Rouser and Neuman.[28] Material suspected of being phosphorylcholine was eluted from chromatograms and subjected to prolonged alkaline hydrolysis followed by identification of choline and phosphoric acid. Choline was run in butanol–water (R_f 0.09) and phenol–water (R_f 0.95) and was detected with the choline reagent described by Chargaff, Levine and Green.[29] This reagent gives little or no color with phosphorylcholine. Phosphoric acid was identified by chromatography in phenol–water (R_f 0.05) and the solvent systems of Hanes and Isherwood. The modified phosphate reagent was used to locate phosphoric acid.

Identification of Sphingosine and Derivatives.—The petroleum ether, chloroform, methanol and aqueous fractions from the alkaline hydrolysis of sphingomyelin were chromatographed in phenol–water. Since no authentic samples of sphingosine phosphate and sphingosinephosphorylcholine were available, it was necessary to elute compounds containing phosphoric acid as shown by the phosphate spray (see above) and possessing a free amino group as shown by the ninhydrin spray (100 mg. of ninhydrin per 100 ml. of *n*-butanol saturated with water) and subject them to complete hydrolysis. After hydrolysis of sphingosine phosphate, both sphingosine and phosphoric acid can be identified. Hydrolysis of sphingosinephosphorylcholine yields both sphingosine and phosphorylcholine which can be readily identified. Paper chromatography in phenol–water yields varying results with sphingosine. When present in small amounts it moves with the solvent front. As the concentration applied on the paper is increased the substance moves increasingly more slowly until a limiting R_f value of 0.82 is reached. The absence of phosphate and the presence of an amino group identifies this compound on chromatograms. Quite unexpectedly, sphingosine was found to give a strong positive test with the choline spray reagent. (A sample of authentic sphingosine was prepared by total hydrolysis of a preparation of cerebroside.) The compound present in the petroleum ether fraction with an R_f value of 0.85 in phenol–water was identified as sphingosine phosphate. Sphingosinephosphorylcholine (R_f in phenol/water 0.65) was found in the chloroform fraction. The movement of sphingosine phosphate and sphingosinephosphorylcholine may be impeded by high concentrations of fatty acid or unhydrolyzed sphingomyelin which spread back from the phenol solvent front. Fatty acid, free or combined, can be detected by dipping in 0.1% Rhodamine B in 50% aqueous ethanol, followed by washing in running tap water to remove excess dye. The white fluorescence in ultraviolet light exhibited by sphingosine phosphate and the bright light-blue of sphingosinephosphorylcholine aid in the identification of these compounds.

Periodate Oxidation.—The method was patterned after the work of Carter and co-workers[9] on sphingosine. One ml. of the petroleum ether fraction (containing sphingosine phosphate) or one ml. of the chloroform fraction (containing sphingosinephosphorylcholine) from the partial hydrolysis of sphingomyelin was shaken vigorously on a shaking machine at room temperature (28°) for 5 hours with 2 ml. of aqueous sodium metaperiodate (30 mg. per ml.). A sample of sphingosine (obtained from the total hydrolysis of cerebroside) was run in the same manner as a control. The organic fractions were checked for aldehydes with the fuchsin sulfurous acid test after the addition of a drop of methanol to a drop of test solution and chromatographed in phenol–water. A positive aldehyde test was always obtained and paper chromatography demonstrated the absence of phosphorylated compounds of sphingosine used as starting materials. The aqueous layer was tested for aldehydes and a positive test was always obtained. Since iodates interfere with the color test for glycolaldehyde and must be removed before paper chromatography, excess acetoin was added to convert periodate to iodate, and iodate was precipitated with excess barium chloride. The small amount of iodate still in solution was sufficient to interfere with the glycolaldehyde test and was converted to iodide with a drop of 1% sulfurous acid solution. The barium was removed before paper chromatography with Dowex-50 resin and the aqueous solutions were frozen and dried to remove excess acid.

Identification of Glycolaldehyde and Glycolaldehyde Phosphate.—A sample of authentic glycolaldehyde phosphate was obtained by the periodate oxidation of α-glycerophosphate at 5° for 24 hours. The aqueous layer from the periodate oxidation of the petroleum ether fraction was compared with the authentic sample and was found to contain a compound which moved to the same location as the authentic sample. The R_f values were in *t*-amyl alcohol–*p*-toluenesulfonic acid–water, 0.25; in *t*-butanol-picric acid–water, 0.25. After elution from chromatograms the compound from the periodate oxidation could be hydrolyzed by dilute alkali to give phosphoric acid and a compound that gave a positive test with the Dische diphenylamine test[30] for glycolaldehyde. The compound obtained from periodate oxidation of the chloroform fraction gave a positive glycolaldehyde color test.

(27) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(28) G. Rouser and W. F. Neuman, *Federation Proc.*, **11**, 268 (1952).

(29) E. Chargaff, C. Levine and C. Green, *J. Biol. Chem.*, **175**, 67 (1948).

(30) Z. Dische and E. Borenfreund, *ibid.*, **180**, 1297 (1949).

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