an authentical sample of nonadecanoic acid. The X-ray diffraction pattern of the acid was identical with that of nonadecanoic acid; long spacing value 45.0 Å. A sample of the acid (17 mg.) was converted into the tribromoanilide, m.p. 126.5–127.5°, which did not depress the melting point of an authentic sample of the tribromoanilide of nonadecanoic acid.

*Anal.* Calcd. for $C_{25}H_{40}ONBr_3$: C, 49.2; H, 6.6. Found: C, 49.3; H, 6.1.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON]

# The Action of Lecithinase D on Lecithin. The Enzymatic Preparation of D-1,2-Dipalmitolein and D-1,2-Dipalmitin[1]

BY DONALD J. HANAHAN AND ROBERT VERCAMER

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The action of lecithinase D of *Cl. perfringens* type A toxin on pure lecithins in 98% ether–2% alcohol has been studied. When (dipalmitoleyl)-L-α-lecithin was used as substrate, D-1,2-dipalmitolein and phosphorylcholine were obtained as products. When the substrate was (dipalmitoyl)-L-α-lecithin, D-1,2-dipalmitin and phosphorylcholine were found. The yield of products was 90% or greater. The reaction proceeded smoothly and reproducibly in this solvent system and was followed by a direct titration of the phosphorylcholine in the reaction medium.

In 1941, MacFarlane and Knight[2] reported that the toxic filtrate of *Clostridium welchii* cultures could attack lecithin with the production of a diglyceride and phosphorylcholine. Since then this enzyme system, called lecithinase D,[3] has been found in *Cl. oedemantiens*,[4] *Cl. bifermentans*,[4] *the* venom of *Bothrops alternatus*,[5] and in the brain tissue of the rabbit, dog and bull.[6] It has been found to be active primarily on lecithin[2] and to a lesser degree on sphingomyelin,[7] and inactive toward cephalin,[7] phosphatidylserine,[7] lysolecithin,[8] cerebrosides[8] and glycerylphosphorylcholine.[8]

Previously it had been shown in this Laboratory[9,10] that the lecithinase A of snake venoms and pancreatin can attack lecithin in solvent systems such as diethyl ether or 95% ether–5% ethyl alcohol. In a continuation of the study of the mode and specificity of action of the phospholipide-hydrolyzing enzymes, we have found that the lecithinase D of the toxic filtrate of *Clostridium perfringens* type A cultures can attack lecithin in solvent systems such as diethyl ether or 98% ether–2% ethyl alcohol. When a pure, unsaturated lecithin, (dipalmitoleyl)-L-α-lecithin is used as a substrate, the only products of the enzymatic action are an asymmetrical, unsaturated diglyceride, D-1,2-dipalmitolein, and phosphorylcholine. Similarly, the saturated lecithin, (dipalmitoyl)-L-α-lecithin yields the asymmetrical, saturated diglyceride, D-1,2-dipalmitin and phosphorylcholine. In both cases, yields of 90% or greater are obtained.

The reaction mixture consisted of a solution of the lecithin in the ether–alcohol solvent to which was added the enzyme solution in water. A homogeneous mixture was obtained and remained so throughout the course of the reaction. When this system was used for the assay of the enzyme activity of a toxic filtrate or a kinetic study on the enzyme action, additional alcohol was added at the end of the incubation period to stop the reaction and to allow for the direct titration of the liberated phosphorylcholine in the reaction mixture with methanolic NaOH to the cresol red end-point. Thus the progress of the hydrolysis could be followed conveniently and reproducibly by this procedure. Previous methods for following this reaction included the determination of the water-soluble phosphate which was formed[2] and a manometric assay of the amount of $CO_2$ liberated from a bicarbonate buffer by the phosphorylcholine.[8] It is felt that the presently described titrimetric procedure represents a less laborious and more accurate means for measurement of the progress of the reaction.

The isolation of the products could be accomplished by the addition of excess water to the reaction mixture. The ether fraction, which contained the diglyceride, was removed and washed with water. When an excess of enzyme was used, it has been our experience that the ether fraction consisted solely of the diglyceride. It is interesting to note that the partition ratio of the intact lecithin between ether and water is apparently in favor of the water, for this compound can be extracted almost quantitatively from the ether by the addition of water. Consequently, any contamination by unreacted substrate is minimal. Phosphorylcholine can be isolated as the calcium salt from the original aqueous fraction and washings.

As had been pointed out by MacFarlane and Knight[2] and confirmed and extended by Zamecnik, *et al.*,[8] calcium ions are apparently the primary activators for this enzyme system. In agreement with these observations, we have found that calcium was necessary for the enzymatic action to

(1) Presented in part before the Division of Biological Chemistry at the 123rd Meeting of the American Chemical Society, Los Angeles, Calif., March 15-20, 1953.

(2) M. G. MacFarlane and B. C. J. G. Knight, *Biochem. J.*, **35**, 884 (1941).

(3) Inasmuch as there is considerable confusion as to the proper nomenclature of these enzymes, we prefer to use the term lecithinase D to designate that enzyme capable of removing phosphorylcholine from lecithin.

(4) M. G. MacFarlane, *Biochem. J.*, **42**, 590 (1948).

(5) J. J. Vidal Breard and V. E. Elias, *Arch. farm. bioquim. Tucuman*, **5**, 77 (1950).

(6) K. V. Druzhinina and M. G. Kritzman, *Biokhimiya*, **17**, 77 (1952).

(7) M. G. MacFarlane, *Biochem. J.*, **42**, 587 (1948).

(8) P. G. Zamecnik, L. E. Brewster and F. Lipmann, *J. Exptl. Med.*, **85**, 381 (1947).

(9) D. J. Hanahan, *J. Biol. Chem.*, **195**, 199 (1952).

(10) D. J. Hanahan, M. Rodbell and L. D. Turner, *J. Biol. Chem.*, in press.

proceed in an ether–alcohol medium. Inasmuch as calcium formed an ether-insoluble complex with both the unsaturated and saturated lecithins, it was necessary to use a 98% ether–2% alcohol solvent system in which the complex was soluble and a completely homogeneous solution was obtained throughout the reaction. An additional reason for the use of this particular solvent was the insolubility of the saturated lecithin in diethyl ether alone. However, if alcohol concentrations greater than 2% were used, the reaction was inhibited.

Variations in substrate concentrations produced the expected changes in the rate of reaction (Fig. 1). Inasmuch as the same reaction rates were obtained for the degradation of the unsaturated as well as the saturated lecithin, it was apparent that this enzyme exhibited no specificity with regard to the fatty acid portion of these molecules. A $K_m$ value of $6.8 \times 10^{-3}$ $M$ was obtained for both substrates. Also any changes in enzyme concentrations produced a proportional change in the rate of reaction. In agreement with previous observations,[7,8] this enzyme system did not attack monopalmitoleyllecithin,[10] monopalmitoyllecithin[10] or yeast cephalin.

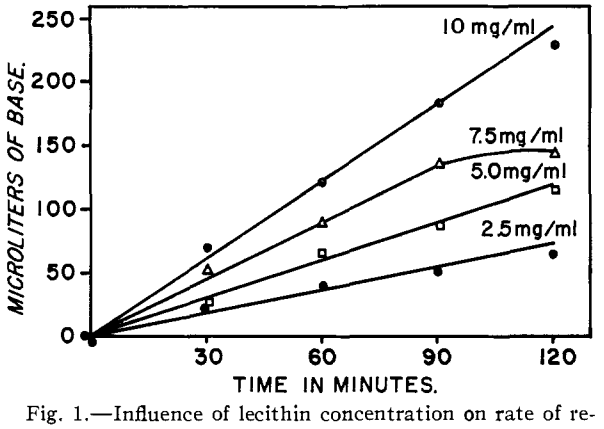


Fig. 1.—Influence of lecithin concentration on rate of reaction of toxin in diethyl ether–alcohol. Reaction mixture contained substrate in 1 ml. of 98% ether–2% ethanol, 10 $\gamma$ of 0.04 $M$ $Ca^{++}$, 40 $\gamma$ of toxin (200–400 MLD/ml.). Exactly same curve as above obtained with either unsaturated or saturated lecithin.

The rather pronounced specificity of this enzyme should make it useful in establishing the structure of lecithins and sphingomyelins, and also aid in the purification of such lipid fractions as cephalins, lysolecithins, etc., by removal of any contaminating lecithin or sphingomyelin.

The infrared spectra of dipalmitolein and dipalmitin are shown in Fig. 2. The unsaturated diglyceride (curve 1, Fig. 2) showed an absorption band at 14.3 $\mu$ which is attributable to the *cis* configuration of the palmitoleic acid attached to the diglyceride. This band was not present in the enzymatically synthesized dipalmitin (Fig. 2, curve 2), or in dipalmitin which was prepared from the unsaturated diglyceride. Many of the characteristic bands associated with triglyceride structure[11] are present in the spectra of both diglycerides: the C–H stretching band at 3.5 $\mu$, the C=O stretching vibration at 5.7 $\mu$, the C–H bending band at 6.9 $\mu$ and the C–O (ester) band at 8.8 $\mu$.

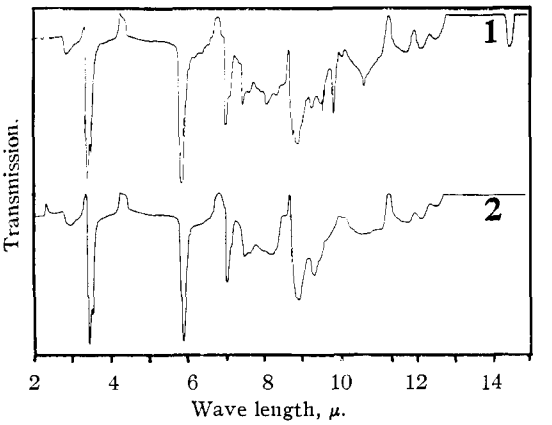


Fig. 2.—Infrared spectra: 1, D-1,2-dipalmitolein, 10.4% in $CHCl_3$, 0.05 mm. cell; 2, D-1,2-dipalmitin, 8.1% in $CHCl_3$, 0.05 mm. cell; Perkin-Elmer Model 21 recording infrared spectrophotometer.

## Experimental

**Materials.**—(Dipalmitoleyl)-L-$\alpha$-lecithin was prepared from fresh commercial bakers' yeast[12] by the chromatographic technique of Hanahan and Jayko.[13] (Dipalmitoyl)-L-$\alpha$-lecithin was prepared by the catalytic hydrogenation at atmospheric pressure of the unsaturated lecithin over platinum oxide.

*Clostridium perfringens* Type A toxin (400–500 MLD per ml.), without preservative, was kindly supplied by Dr. H. D. Piersma of the Lederle Laboratories.

0.02 $N$ NaOH in 90% methanol was prepared essentially by the procedure of Stetten and Grail.[14] A 0.1% solution of cresol red, neutralized with NaOH, was employed as an indicator. Diethyl ether was freshly distilled from sodium.

**Assay System.**—A typical reaction was run as follows: 10 mg. of substrate[15] was dissolved in 1.0 ml. of 98% ether–2% ethyl alcohol in a 2-ml. volumetric flask (straight side type), 0.02 ml. of 0.04 $M$ $CaCl_2$ was added and the solution mixed well by shaking. Then 0.04 ml. of toxin at *p*H 7.0 was added, the solution mixed again and incubated at room temperature. After the desired incubation period, 1.0 ml. of 95% ethyl alcohol and 0.02 ml. of cresol red indicator were added and the free phosphorylcholine titrated with 0.02 $N$ NaOH using a Gilmont Ultramicroburet. During the titration, nitrogen was bubbled through the solution to exclude any $CO_2$ and also to stir the solution.

Samples of pure phosphorylcholine were titrated in the same solvent system with and without lecithin being present. Recoveries of 99–100% were obtained on amounts of phosphorylcholine as low as 0.1 mg.

**Enzymatic Preparation of Diglycerides. D-1,2-Dipalmitolein.**—One gram of (dipalmitoleyl)-L-$\alpha$-lecithin was dissolved in 100 ml. of 98% ether–2% ethyl alcohol and to this solution was added 2 ml of 0.02 $M$ $CaCl_2$ and 5 ml. of toxin at *p*H 7.0. The solution was mixed well by shaking and allowed to stand at room temperature for 3 hours. The reaction mixture was transferred to a separatory funnel, 25 ml. of water was added and the contents mixed well. The aqueous phase was removed and saved for phosphorylcholine isolation. The ether fraction was washed three times with 25-ml. portions of water. All the water washings were saved and combined with the original aqueous fraction.

(11) O. D. Shreve, M. R. Heether, H. B. Knight and D. Swern, *Anal. Chem.*, **22**, 1498 (1950).

(12) This yeast was generously supplied by Standard Brands, Inc., Sumner, Wash.

(13) D. J. Hanahan and M. E. Jayko, THIS JOURNAL, **74**, 5070 (1952).

(14) D. W. Stetten, Jr., and G. F. Grail, *Ind. Eng. Chem., Anal. Ed.*, **15**, 300 (1943).

(15) The solution of the saturated lecithin in this solvent is best accomplished by first dissolving the compound in the alcohol, then diluting to volume with ether.

The ether solution was dried over anhydrous $Na_2SO_4$ and then evaporated to dryness at room temperature under a stream of nitrogen; yield 650 mg. of a colorless oil (theory 677 mg.). Calcd. for dipalmitolein: hydrogen number,[16] 282; sapn. equiv., 282.5. Found: hydrogen number, 282; sapn. equiv., 282; $[\alpha]^{25}D$ −2.3° (*c* 12.0 in $CHCl_3$).

Hydrogenation of this unsaturated compound gave as the only product, D-1,2-dipalmitin, m.p. 67–68° (reported value[17] 67–67.5°). Alkaline hydrolysis of 350 mg. of the unsaturated diglyceride, followed by acidification and extraction with diethyl ether yielded 300 mg. of palmitoleic acid (theory 316 mg.). Calcd. for palmitoleic acid: neut. equiv., 254.0; hydrogen number, 254.0. Found: neut. equiv., 255.0; hydrogen number, 253.5. Fifty milligrams of the acid was converted to the amide[10]; yield 42 mg., m.p. 68.5–69.5°. Admixture with pure palmitoleamide[10] did not alter the melting point.

Hydroxylation of 50 mg. of the unsaturated fatty acid by the procedure of Swern, *et al.*,[18] gave 40 mg. of *cis*-dihydroxypalmitic acid, m.p. 85–86° (reported value[10] 85–86°).

The infrared pattern of the free unsaturated fatty acid was identical with that reported for palmitoleic acid.[13]

**D-1,2-Dipalmitin.**—Under exactly the same conditions as described above for the preparation of the unsaturated diglyceride, the saturated diglyceride may be obtained from (dipalmitoyl)-L-α-lecithin. In a typical run, 625 mg. of dipalmitin was obtained from 1.00 g. of saturated lecithin. The residue from the evaporation of the ether-soluble fraction (see above) was crystallized three times from $CHCl_3$–petroleum ether (1:10); yield 590 mg., m.p. 67.5–68° (reported value[17] 67–67.5°).

*Anal.*[19] Calcd. for $C_{35}H_{68}O_5$ (568): C, 73.8; H, 12.05. Found: C, 73.75; H, 12.13; $[\alpha]^{25}D$ −2.3° (*c* 11.7 in $CHCl_3$), reported value[17] $[\alpha]^{25}D$ −2.3°, in $CHCl_3$.

*p*-Nitrobenzoyl derivative, m.p. 60–61° (reported value[17] 60–60.5°). Hydrolysis of 100 mg. of this diglyceride under the same conditions as for the unsaturated diglyceride gave 75 mg. of fatty acid (theory 80 mg.). After recrystallization three times from 60% ethanol, 60 mg. of acid, m.p. 60–61°, was obtained which when admixed with an authentic sample of palmitic acid had m.p. 60–61°; neut. equiv. 256.0 (theory for palmitic acid 256.0).

**Phosphorylcholine.**—The combined original aqueous fraction and the washings were concentrated to dryness and the calcium salt of phosphorylcholine was isolated in 90% yields, essentially by the procedure described by MacFarlane and Knight.[2]

*Anal.* Calcd. for $C_5H_{13}O_4NPClCa5H_2O$ (348): P, 8.91; N, 4.02. Found: P, 8.87; N, 4.10.

**Addenda.**—After the completion of this manuscript, a communication by C. Long and M. F. Maguire appeared in *Biochem. J.*, **55**, xv (1953), wherein they report that *Cl. welchii* type A α-toxin acts as expected on ovolecithin but exhibited no activity toward saturated lecithins in an aqueous medium.

(16) Hydrogen number = wt. of sample in mg. × 2.4/ml. of hydrogen absorbed (S.T.P.).

(17) J. C. Sowden and H. O. L. Fischer, THIS JOURNAL, **63**, 3244 (1941).

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

(19) Analyzed by the Elek Micro Analytical Laboratories, 4763 W. Adams Blvd., Los Angeles, Calif.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY]

# The Synthesis of Peptides Related to Gramicidin S

BY BERNARD F. ERLANGER,[1] HOWARD SACHS AND ERWIN BRAND[2]

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The synthesis of a crystalline decapeptide containing the same sequence and configurations of amino acids as exist in gramicidin S is described. The synthetic methods were chosen so as to preclude the possibility of the formation of mixtures of diastereoisomers.

We are reporting the synthesis of a decapeptide having the sequence and configurations of amino acids believed to exist in gramicidin S. Consden, Gordon, Martin and Synge[3] have determined the sequence of the five different amino acid residues of gramicidin S to be[4]: -Val-Orn-Leu-Phe-Pro- (L-L-L-D-L). It has been established by Battersby and Craig[5] that the molecular weight of gramicidin S is that of a decapeptide. Thus the sequence must appear twice. Sanger[6] has shown that the only free amino group is the δ-amino group of ornithine. This result and X-ray analysis[7] support the hypothesis that gramicidin S is a cyclic decapeptide.

Harris and Work[8] have synthesized three pentapeptide derivatives having the sequence of gramicidin S: *p*-Tos·Val-Orn-Leu-Phe-Pro·OMe·HCl (L-L-L-D-L)[9]; *p*-Tos·Val-Orn-Leu-Phe-Pro·$NH_2$·HCl (L-L-L-D-L); H·Val-Orn-Leu-Phe-Pro·OMe·2HCl (L-L-L-D-L). The last peptide could not be crystallized and no analysis or physical data were reported. The synthetic techniques used were those developed by Bergmann and his collaborators.[10]

Recently, Schumann and Boissonas[11] reported the synthesis of H·Val-Z·Orn-Leu-Phe-Pro·OH (L-L-L-D-L), an intermediate which could be useful for the synthesis of the decapeptide. They used, exclusively, the mixed anhydride method of peptide synthesis devised by Boissonnas[12] and by Vaughan and Osato.[13] They employed phthaloyl[14–16] as a protecting group.

It has been found by Vaughan[17] and by the au-

(1) Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York City.

(2) Deceased July 11, 1953.

(3) R. Consden, A. Gordon, A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **41**, 596 (1947).

(4) For key to abbreviations, see footnotes to Table I and B. F. Erlanger and E. Brand, THIS JOURNAL, **73**, 3508 (1951).

(5) A. R. Battersby and L. C. Craig, *ibid.*, **73**, 1887 (1951).

(6) F. Sanger, *Biochem. J.*, **40**, 261 (1946).

(7) D. C. Hodgkin, *Cold Spring Harbor Symposia*, **14**, 65 (1949).

(8) J. I. Harris and T. S. Work, *Biochem. J.*, **46**, 196, 582 (1950).

(9) *p*-Tos = *p*-toluenesulfonyl.

(10) For references *cf.* J. S. Fruton, *Adv. Prot. Chem.*, **5**, 1 (1949).

(11) I. Schumann and R. A. Boissonnas, *Helv. Chim. Acta*, **35**, 2237 (1952).

(12) R. A. Boissonnas, *ibid.*, **35**, 874 (1951).

(13) J. R. Vaughan and R. L. Osato, THIS JOURNAL, **73**, 5553 (1951); **74**, 676 (1952).

(14) J. C. Sheehan and V. S. Frank, *ibid.*, **71**, 1856 (1949).

(15) F. E. King and D. A. A. Kidd, *J. Chem. Soc.*, 3315 (1949).

(16) F. E. King, B. S. Jackson and D. A. A. Kidd, *ibid.*, 243 (1951).

(17) J. R. Vaughan, THIS JOURNAL, **74**, 6137 (1952).