L-α-(Dioleoyl)lecithin.* An Alternate Route to Its Synthesis

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L-α-(dioleoyl)lecithin has been prepared by phosphorylation of D-α,β-diolein with phosphorus oxychloride and quinoline, esterification of the resulting dioleoyl L-α-glycerophosphoric acid dichloride with choline chloride or choline iodide in the presence of pyridine, and separation of the reaction products by means of column chromatography on silicic acid. The L-α-(dioleoyl)lecithin was obtained in yields ranging from 44% to 50% of theory and was identical in every respect with L-α-(dioleoyl)lecithin obtained by our other methods.

The synthesis of several naturally occurring glycerolphosphatides and the elucidation of their structure and configuration has been reported in a series of publications from this laboratory (Baer and co-workers, 1939, 1950, 1952, 1953, 1955 a and b). Six years ago Baer *et al.* (1956) described the first synthesis of a lecithin containing two identical unsaturated fatty acid substituents, namely L-α-(dioleoyl)lecithin. The lecithin, which possesses the structure and configuration of naturally occurring phospholipids, was obtained from D-acetone glycerol via the following intermediates: acetone L-α-glycerylphenylphosphoryl chloride → acetone L-α-glycerylphenylphosphoryl ethylene chlorohydrin → L-α-glycerylphosphoryl ethylene chlorohydrin → L-α-dioleoylglycerylphosphoryl ethylene chlorohydrin → L-α-(dioleoyl)lecithin. Shortly afterwards Tattrie and McArthur (1957) reported a method for the preparation of L-α-lecithins containing two identical saturated or unsaturated fatty acid substituents by the acylation of L-α-glycerylphosphorylcholine (GPC) with a large excess of acyl chloride in the absence of an inorganic base. It is unfortunate that this method in spite of its promise has two serious shortcomings. Not only is the acylation of L-α-glycerylphosphorylcholine extremely time-consuming—it may require from 2 to 33 days, depending somewhat on the length of the fatty acid to be introduced (Kögl *et al.*, 1960)—but also it gives rise to the formation of considerable amounts of lysolecithins (Kögl *et al.*, 1960). Both disadvantages were overcome and the lecithins were made readily accessible from glycerylphosphorylcholine by the discovery that the acylation of its cadmium chloride complex proceeds much more rapidly, even at low temperatures (0°), than the acylation of glycerylphosphorylcholine itself (Baer and Buchnea, 1959b). The acylation of the cadmium chloride complex is essentially finished in less than 2 hours. The lecithins are obtained in good yields and as optically pure compounds provided optically pure L-α-glycerylphosphorylcholine is used as starting material. Starting with synthetic L-α-glycerylphosphorylcholine, Baer and Buchnea (1959b) prepared by this method L-α-(dioleoyl)-lecithin, L-α-(distearoyl)lecithin, and L-α-(dimyristoyl)lecithin, which they found to be identical in every respect with the corresponding compounds obtained in this laboratory by other methods (Baer and Kates, 1950; Baer and Maurukas, 1952; Baer *et al.*, 1952, 1956).

To make the synthesis of lecithins from glycerylphosphorylcholine more attractive than by other methods, it was necessary to make L-α-glycerylphosphorylcholine readily accessible. Tattrie and McArthur (1955) accomplished this by developing a procedure for preparation of L-α-glycerylphosphorylcholine from egg-yolk lecithin via catalytic deacylation with mercuric chloride. Investigations by Baer and Buchnea (1959b) revealed, however, that L-α-glycerylphosphorylcholine prepared by this method unfortunately is not a suitable material for the synthesis of L-α-lecithins if they are to be obtained as optically pure compounds. This was fully confirmed, although inadvertently, by other investigators who have tried to use this material for the synthesis of optically pure L-α-lecithins. Thus Kögl *et al.* (1960), using the original methods of Tattrie and McArthur both for the preparation of L-α-glycerylphosphorylcholine from egg-yolk lecithin and for its reacylation, have prepared L-α-(dioleoyl)lecithin as well as a series of eight saturated lecithins containing fatty acid substituents with chains ranging from 2 to 22 carbon atoms, but not one of them possessed the structural and optical purity of members of a similar series of lecithins (M_D mean value +50.1°) prepared in this laboratory by different methods (Baer and co-workers, 1950, 1952, 1956, 1959c). In fact the molecular rotations reported by Kögl *et al.* (1960) for their series of L-α-lecithins, which range from +44.6° to +40.8°, suggest that they are mixtures containing from 11 to 18% of DL-α- or β-lecithins. Because of the possible great value of Tattrie and McArthur's procedure for the preparation of L-α-glycerylphosphorylcholine from egg-yolk, various attempts were made in

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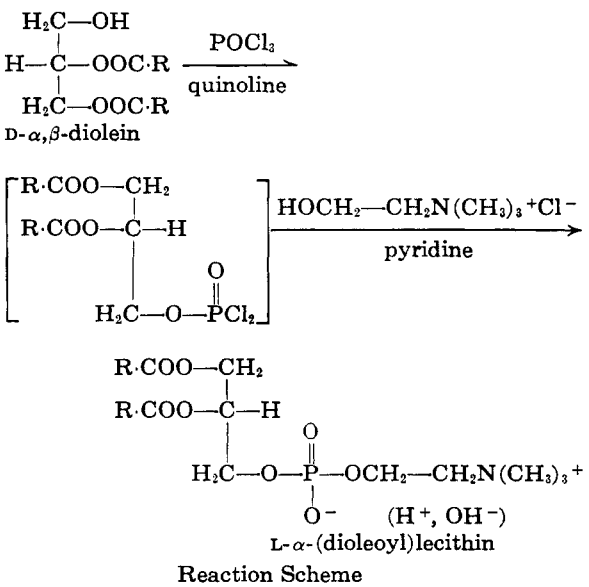
this laboratory to modify the procedure in such a manner that it would yield L-α-glycerylphosphorylcholine which would be suitable for the preparation of optically pure L-α-lecithins. These attempts were unsuccessful. More recently, however, Urakami and Okura (1958) and Urakami *et al.* (1960) have shown that if the deacylation of egg-yolk lecithin is carried out with lithium aluminum hydride an L-α-glycerylphosphorylcholine is obtained which on reacylation yields optically pure L-α-lecithins. Material obtained by this method was used successfully by de Haas and van Deenen (1960) for the preparation of optically pure L-α-(dioleoyl)lecithin. Recently, L-α-glycerylphosphorylcholine has become more readily accessible also by the synthetic procedure of Baer and Kates (1948), through shortcuts introduced in this procedure by Maurukas and Holland (1961).

We wish now to report yet another procedure by means of which we have obtained L-α-(dioleoyl)-lecithin, and which it is hoped may prove to be applicable also to the synthesis of other lecithins. It resembles our earlier procedure for the synthesis of L-α-lecithins from D-α,β-diglycerides, except that phenylphosphoryl dichloride is replaced by phosphorus oxychloride, and the necessity of removing the protective phenyl group by catalytic hydrogenolysis is thus avoided. At one time



Reaction Scheme

phosphorus oxychloride was widely used as a phosphorylating agent. In recent years, however, its mono- and diphenyl- or benzyl derivatives were preferred, as they yield fewer by-products. With the introduction of chromatographic procedures for the separation of phosphate esters, phosphorus oxychloride once again has come into favor as a phosphorylating agent, especially for the preparation of unsaturated phosphatides. It has been used successfully in recent years by Baer and Buchnea for the synthesis of dioleoyl L-α-glycerylphosphoric acid (1958a), bis(dioleoyl-L-α-glyceryl) phosphoric acid (1958a), (dioleoyl-L-α-glycerylphosphoryl)-L-α-glycerol (1958b), L-α-(dioleoyl)cephalin (1959a), and mixed-acid L-α-cephalins (1961) containing oleic acid.

The synthesis of L-α-(dioleoyl)lecithin by the new procedure is as follows (see Reaction Scheme): D-α,β-diolein is phosphorylated with phosphorus oxychloride in the presence of quinoline, the resulting phosphatidic acid chloride is esterified with choline chloride or choline iodide in the presence of pyridine, and the reaction mixture is separated by column chromatography on silicic acid. The L-α-(dioleoyl)lecithin, a slightly colored wax-like material, was obtained in yields ranging from 44–50% and was identical in every respect with L-α-(dioleoyl)lecithin prepared by our two previous methods.

Experimental Procedures and Results

Materials.—Anhydrous quinoline, pyridine, and ethanol-free chloroform were prepared as described in an earlier publication by Baer and Buchnea (1959a). Commercial choline chloride was purified by recrystallization from anhydrous ethanol. It was finely powdered under anhydrous conditions, and was dried in a vacuum of 0.1 mm at 56° over phosphorus pentoxide. Only the amount necessary for the immediate experiment was dried, and it was transferred to the reaction vessel with the exclusion of moisture. The silicic acid was from Mallinckrodt 100 mesh (powder), analytical reagent. The silicic acid was sifted and all particles going through a sieve of 150 mesh per linear inch were removed. The oleoyl chloride was prepared from pure (99.9%) oleic acid (Rubin and Paisley, 1960) by means of oxalyl chloride (Wood *et al.*, 1944). The D-α,β-diolein was obtained by the method of Baer and Buchnea (1958a,b).

Choline Iodide.—Fifty millimoles (7.0 g) of choline chloride were dissolved in 70 ml of 99% ethanol, and to the solution was added a solution of 50 mmoles (7.5 g) of sodium iodide in 75 ml of 99% ethanol. The mixture was filtered, and to the filtrate was added anhydrous ether until no further precipitation occurred. The precipitate was collected on a Buchner funnel, washed with ether, and dried *in vacuo*. The choline iodide was obtained in a yield of 75% of theory. It was finely powdered and sifted through a sieve with 150 mesh per linear inch. *Anal.* Calcd. for $C_5H_{14}ONI$ (231.1): N, 6.06. Found: 5.91.

L-α-(Dioleoyl)lecithin.—Into a carefully dried 50-ml three-necked round flask with ground joints, and equipped with an oil-sealed stirrer, calcium chloride tube, and dropping funnel, was placed 0.37 ml (4.0 mmoles) of freshly distilled phosphorus oxychloride. The flask was immersed in an ice-bath, and to the phosphorus oxychloride was added with vigorous stirring a solution of 2.48 g (4.0 mmoles) of D-α,β-diolein and 0.52 ml (4.4 mmoles) of anhydrous quinoline in 20 ml of anhy-

drous and ethanol-free chloroform in the course of 90 minutes. The cold bath then was replaced by a water-bath at 35°, and the reaction mixture was kept at this temperature for 1 hour. At the end of this period, 5 ml of anhydrous pyridine, 0.63 g (4.5 mmoles) of finely powdered and thoroughly dried choline chloride, and 15 ml of glass beads (approximately 4 mm in diameter) were added quickly under anhydrous conditions, and the mixture was stirred at room temperature (20°–25°) until the choline chloride had gone into solution. Approximately 20 hours were required. At this point 0.1 ml of water was added and the stirring was continued for 30 minutes. The reaction mixture then was filtered, the glass beads were rinsed with three 15-ml portions of chloroform, and the combined filtrates were evaporated under diminished pressure from a bath at 30°–35°. The residue was taken up in 100 ml of anhydrous ether, and the solution was cleared by centrifugation. The precipitate was extracted with three 20-ml portions of ether. The combined ether solutions were washed as rapidly as possible with two 50-ml portions of ice-cold 0.1 N sulfuric acid,[1] one 25-ml portion of a saturated sodium bicarbonate solution, and two 25-ml portions of water. During washing with sodium bicarbonate, emulsions form which are readily destroyed by centrifugation. The ether solution was concentrated by distillation under reduced pressure, and the residue was freed of solvent in a vacuum of 0.1 mm at a bath temperature of 30°–35°. The remaining material was dissolved in 125 ml of 95% ethanol. To the solution was added 1.5 g of finely powdered silver carbonate, and the mixture was shaken for 30 minutes. The silver salts were removed by centrifugation, the precipitate was extracted with two 20-ml portions of 95% ethanol, and the combined ethanolic solutions were passed through a column (1.5 × 20 cm) of amberlite IRC-50 (H^+). The amberlite column was rinsed with 95% ethanol followed by ether. The combined effluents were evaporated under reduced pressure, and the residue was dried in a vacuum of 0.1 mm at 30°–35°. The wax-like, slightly yellowish material, weighing 3.2 g, was purified by passing its solution in 25 ml of chloroform through a column (3.2 × 30 cm) of silicic acid (Mallinckrodt), washing the column first with chloroform and then with a mixture of chloroform and methanol (4:1, v/v) until both eluates were free of solute, and recovering the lecithin with a mixture of chloroform and methanol (3:2, v/v). The lecithin solution was freed of solvents by distillation under reduced pressure, and the residue, an almost colorless wax-like material, was dried in a vacuum of 0.1 mm at a bath temperature of 30°–35° until its weight was constant. The L-α-(dioleoyl)lecithin, a chromatographically homogeneous substance, weighed 1.4 g (yield 44% of theory). It was readily soluble at room temperature in ether, methanol, ethanol, chloroform, and 90% acetone, and moderately soluble in warm petroleum ether (b.p. 35°–60°). $[\alpha]^{26}D$ +6.1° (*c*, 1.5) and $[\alpha]^{28}D$ +6.0° (*c*, 5) in chloroform-methanol (1:1, v/v). Reported $[\alpha]^{25}_D$ + 6.2° (*c*, 5) and 6.1° (*c*, 10) in chloroform-methanol (1:1, v/v). The infrared spectrum of the L-α-(dioleoyl)lecithin was identical with that reported by us for L-α-(dioleoyl)lecithin prepared by a different method (1956). *Anal.* Calcd. for $C_{44}H_{86}O_9NP$ (804.2): N, 1.74; P, 3.85. Found:[2] N, 1.65, 1.68, 1.69; P, 3.81, 3.75, 3.79.

By replacing in the above procedure the strongly hygroscopic choline chloride by an equimolecular amount of finely powdered, nonhygroscopic choline iodide, pure L-α-(dioleoyl)lecithin was obtained in a yield of 50% of theory. *Anal.* Calcd. for $C_{44}H_{86}O_9NP$ (804.2): N, 1.74; P, 3.85. Found: N, 1.69; P, 3.81.

[1] It is essential that the washing with sulfuric acid be carried out as rapidly as possible to avoid loss of lecithin by acid hydrolysis. If the lecithin is being prepared in batches larger than reported here, it is advisable to divide the ether solution in several portions of 150 ml each.

[2] Analytical values for three independent preparations of L-α-(dioleoyl)lecithin.

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A Study of the Purification and Properties of the Phospholipase A of *Crotalus adamanteus* Venom*

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Two proteins (I and II) with phospholipase A activity have been isolated from the venom of *Crotalus adamanteus* (Eastern diamond back rattlesnake) by a procedure involving *p*H change, heat treatment, and subsequent chromatography on diethylaminoethyl-cellulose. Proteins I and II were chromatographically separable and had similar sedimentation constants but differed significantly in their electrophoretic mobility and isoelectric points. The purity of the proteins I and II was calculated to be 80 to 85% and the molecular weight estimated to be in the range of 30–35,000. No protease, nucleotidase, phosphodiesterase, or phosphomonoesterase activity was demonstrable in these purified fractions. Although the specific activity of I and II was not similar, there was no observed difference in the nature of the fatty acids liberated by their action on native ovolecithin. No specificity toward the chain length or unsaturation was evident. The presence of ethylenediaminetetraacetic acid was required throughout the purification for the preservation of maximal activity of these enzymes. In evaluating the mode of action of proteins I and II on lecithin in an ether solution, the inclusion of NaCl, $CaCl_2$, and ethylenediaminetetraacetic acid was found to be necessary for optimum enzymatic activity.

It has been well documented that several different species of snake venoms are potent sources of the enzyme phospholipase A (Kates, 1960). This enzyme has been shown to effect the hydrolytic cleavage of the fatty acid ester at the β or 2 position of L-α-lecithins with the formation of an α'-acyl-L-α-glycerylphosphorylcholine (lysolecithin) and free fatty acid (Tattrie, 1959; Hanahan *et al.*, 1960; de Haas *et al.*, 1960; de Haas and van Deenen, 1961).

Although there have been several reports on the purification of phospholipase A (Radominski and Deichmann, 1958; Neumann and Habermann, 1955; Boman and Kaletta, 1957; Yang *et al.*, 1959a,b), many of the described assay systems have depended upon quite indirect and inexact techniques for the evaluation of enzyme activity, namely, hemolysis and neurotoxic activity. Recently, however, Kawichi and coworkers (Wakui and Kawichi, 1961; Iwanaga and Kawichi, 1959; Wakui and Kawichi, 1959) have described the purification and properties of phospholipase A in certain Japanese and Formosan snake venoms. In this informative study, in which two separate, active phospholipase A fractions were isolated, the assay system depended on a combined enzymatic and chemical determination of lysolecithin formed in the reaction. This present communication confirms in part the results of Kawichi and collaborators and presents additional information and observations on the purification and properties of the phospholipase A of *Crotalus adamanteus* (Eastern diamond back rattlesnake) venom.

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EXPERIMENTAL[1]

Materials.—Dehydrated *Crotalus adamanteus* (Eastern diamond back rattlesnake) venom was purchased from Ross Allen's Reptile Institute, Silver Spring, Fla. The ovolecithin (hen) was prepared by aluminum oxide (Rhodes and Lea, 1957) and silicic acid (Hanahan *et al.*, 1957)

[1] The following abbreviations are used: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid (Na salt); AMP, adenosine-5′-phosphate; FAD, flavin adenine dinucleotide.