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The stereospecific action of phospholipase A on β-lecithins

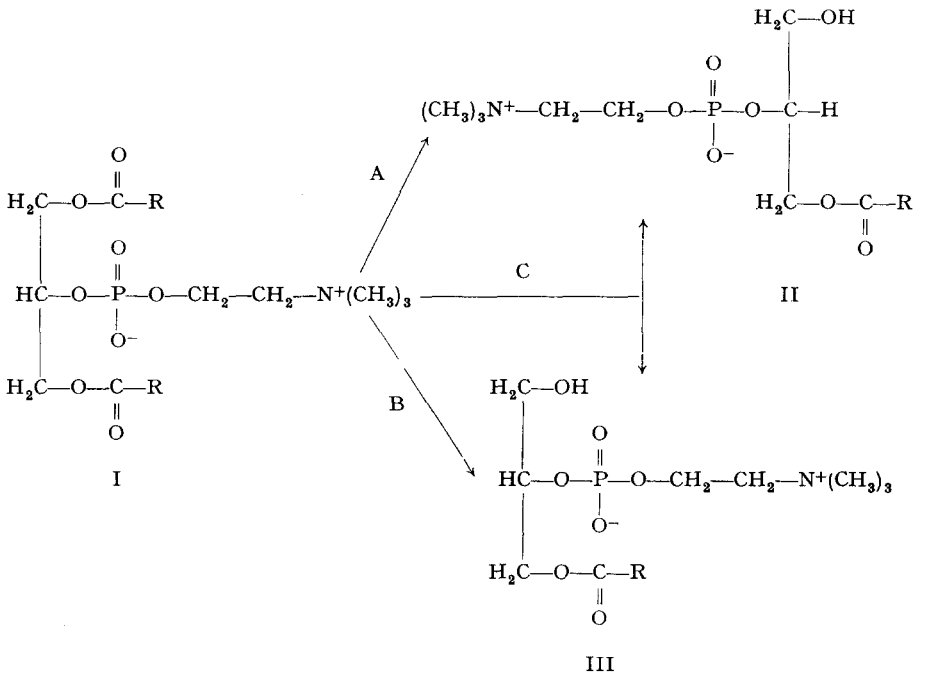
A recent study on the substrate specificity of phospholipase A (phosphatide acylhydrolase, EC 3.1.1.4) demonstrated that the minimal substrate requirements for this enzyme from snake venom (*Crotalus adamantheus*) involve the presence of only one fatty acid, esterified at a position adjacent to the ester linkage between the alcohol and phosphoryl moieties[1]. This concept is in accordance with the prevailing view that phospholipase A catalyses exclusively the cleavage of the β-fatty acid from the L-α-phosphoglyceride molecule[2–6]. These developments did raise doubt to previous reports[7,8] on the substrate inactivity of β-lecithins (I), having the phosphorylcholine moiety attached to position 2 of the glycerol constituent. Actually, a re-investigation showed that a symmetric β-lecithin, when emulsified by ultrasonic irradiation in an aqueous medium, was susceptible to the action of this enzyme[1]. A detailed study of this reaction may contribute to the further elucidation of the stereochemical features of the interaction between substrates and phospholipase A, since β-phospholipids contain a molecular arrangement related to but different from that of natural phospholipids. Taking into consideration the concept of OGSTON[9] an asymmetric degradation of β-lecithin was to be expected.

Starting from α,γ-distearin a specimen of α,γ-distearoyl-β-glycerylphosphorylcholine was synthesized according to methods outlined by HIRT AND BERCHTOLD[10]. An amount of 10 mg of this β-lecithin, emulsified in 1 ml of borate buffer (pH 7) containing 2.5 mM calcium acetate together with 4 mg of venom from *Crotalus adamantheus*, was incubated for 5 h at 37° to give a complete breakdown of the β-lecithin. Quantitative determinations, using chromatography on silica-impregnated paper, showed 1 equiv of a lyso derivative to be produced. Continuation of the incubation for 48 h did not give rise to the formation of any detectable quantities of glycerylphosphorylcholine. Hence, phospholipase A from *Crotalus adamantheus* venom catalyses the liberation of one fatty acid only from the β-lecithin molecule. Considering the various possibilities of breakdown (see scheme), this result already indicates that a non-stereospecific degradation (Pathway C) probably does not occur. The latter mode of action would primarily cause the formation of lyso-β-lecithin in a racemic form (II + III), and on account of the previously observed[1] susceptibility of β-monoacyl-L-α-glycerophosphatides and glycol analogs to phospholipase A a further breakdown into β-glycerylphosphorylcholine should be expected.

The conclusion that the hydrolytic action of phospholipase A is governed by steric conditions (Pathway A or B) was further proved by demonstrating the formed lysolecithin to be optically active. An amount of 1.0 g of β-lecithin was subjected to hydrolysis by phospholipase A on a correspondingly larger scale. After an incubation of 20 h the breakdown was practically complete; the reaction mixture was subjected to lyophilization and extracted with chloroform–methanol (3:1, v/v). The desired lysolecithin was isolated in a pure state in a yield of 70 % by chromatography on a silica column using mixtures of chloroform and methanol as eluents. The colourless powder of α-stearoyl-β-glycerylphosphorylcholine, m.p. 243–245°, was analysed as the $CdCl_2$ adduct. [Found: C, 38.3; H, 7.1; N, 1.7; P, 3.7. $(C_{26}H_{56}NO_8P)_2(CdCl_2)_3$ requires: C, 38.23; H, 6.91; N, 1.71; P, 3.79.]

The optical rotation, *viz.* $[\alpha]_{578}^{20} = +1.45°$ [*c*, 10 in chloroform–methanol (9:1 v/v)], indicates that only one stereo isomer (II or III) was enzymically produced

from the symmetric β-lecithin. Theoretical considerations led us to conclude, tentatively, that the L-isomer (II, Pathway A) has been formed, but the final assignation of the L- or D-configuration must await further degradation experiments on this lysolecithin. The value of the optical rotation already demonstrates that the phosphorylcholine moiety was not subject to migration, the optical activity of *e.g.* γ-stearoyl L-α-glycerylphosphorylcholine being $[\alpha]_D^{20} = -3.2°$.



Confirmatory evidence for the observed stereospecificity of phospholipase A was obtained from experiments involving a re-acylation of the lyso-β-lecithin with a second but different fatty acid, giving an asymmetric β-lecithin. An alcoholic solution of the isolated stereo isomer of α-stearoyl-β-glycerylphosphorylcholine was treated with $CdCl_2$ and the $CdCl_2$ adduct obtained was converted to α-stearoyl-γ-margaroyl-β-glycerylphosphorylcholine according to established procedures[11,12]. This compound was isolated in a pure state in a yield of 45 % after chromatography on silicic acid, while an equal quantity of starting material was recovered as well. Gas–liquid chromatography showed equimolar amounts of stearic and margaric acid to be present. The colourless α-stearoyl-γ-margaroyl-β-glycerylphosphorylcholine, m.p. 233–234°, was shown to possess an optical rotation $[\alpha]_{578}^{20} = 0$. [Found: C, 48.6; H, 8.4; N, 1.6; P, 3.1. $(C_{43}H_{88}NO_9P)_2(CdCl_2)_3$ requires: C, 48.31; H, 8.30; N, 1.31; P, 2.9.]

That this mixed-acid β-lecithin, containing an asymmetric centre, exhibits no measurable optical rotation can be expected on account of the close resemblance between both fatty acid constituents. The stereospecific features of snake-venom phospholipase A were conclusively confirmed by the results obtained on the enzymic hydrolysis of this stereo isomer of α-stearoyl-γ-margaroyl-β-glycerylphosphorylcholine.

After a complete degradation of this substrate the liberated fatty acid was shown by gas–liquid chromatography to consist exclusively of margaric acid, while the produced lysolecithin, which was isolated, contained stearic acid only. This compound turned out to possess an optical rotation, $[\alpha]_{578}^{20} = +1.45°$ [*c*, 10 in chloroform–methanol (9:1, v/v)], being identical to the value of the stereo isomer of α-stearoyl-β-glycerylphosphorylcholine, used as starting material for the preparation of the asymmetric β-lecithin. This finding indicates that no migration occurred neither during the acylation procedure or during the enzymic hydrolysis.

The foregoing experiments repeatedly showed that phospholipase A does catalyse the release of one fatty acid from the β-lecithin molecule with the formation of optical active α-acyl-β-glycerylphosphorylcholine. This stereospecific breakdown supports previous observations indicating that synthetic D-α-lecithins do resist the action of phospholipase A (refs. 4, 7, 8). Hence, it can be concluded that phospholipase A catalyses the hydrolysis of the fatty acid ester linkage, located adjacent to the glycerylphosphoric acid ester bond, only when the fatty acid to be released is allowed to occupy a certain sterical position during the substrate–enzyme interaction.

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