Phospholipase A_2 Hydrolysis of Phospholipids: Use of ^{31}P NMR to Study the Hydrolysis, Acyl Migration, Regiospecific Synthesis, and Solubilization of Phospholipids

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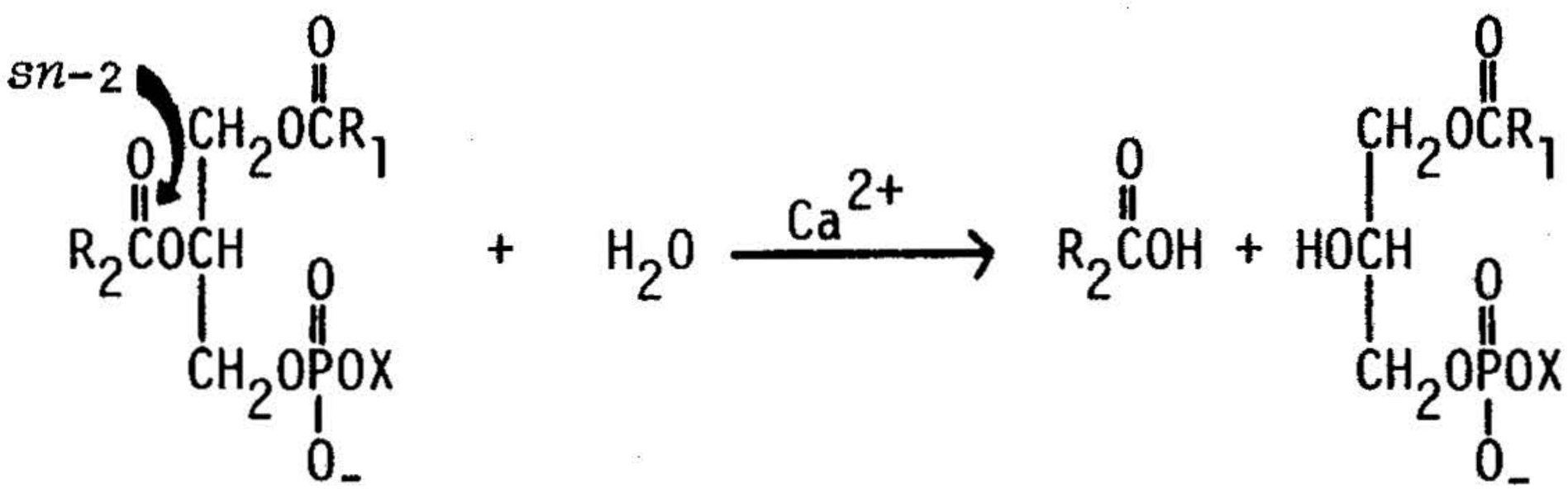
Our laboratory has been studying the kinetics (1) and mechanism of action (2) of phospholipase A_2 which catalyzes the hydrolysis of the fatty acyl chain in the *sn*-2 position of phospholipids to give the 1-acyl lyso-phospholipid product:



Recently, we found that phosphorylcholine-containing lipids activate the enzyme from cobra venom (*Naja naja naja*) toward phosphatidylethanolamine (PE) (3,4). These studies led to the suggestion of two sites for the enzyme - an activator site with minimum specificity for phosphorylcholine and a hydrophobic chain and a catalytic site with less specificity for the polar group. We have now extended these studies to synthetic phospholipids that contain short chain fatty acyl groups and which are water soluble, such as dibutyryl and dihexanoyl phosphatidylcholine (PC). These phospholipids are monomeric below their critical micelle concentration (cmc), yet activate the enzyme. In order to carry out kinetic studies, the long chain phospholipid substrate must generally be solubilized by a detergent such as Triton X-100 which serves as an inert matrix. Further understanding of the mechanism of the activation by short-chain phospholipids requires first a quantitation of the solubilization of these compounds by detergent:

To solve this problem, the very high sensitivity of the ^{31}P-NMR chemical shift to changes in the hydrophobicity of the environment has proven to be very useful. The sensitivity of the chemica shift to structural changes in the phospholipid molecule several atoms away allowed us to also examine several other mechanistic questions including the hydrolysis and specificity of phospholipas

A_2 in mixed phospholipid systems and the kinetics and migration of the acyl and phosphorus group of the lyso-phospholipid products. The new ^{31}P-NMR results also allow us to directly establish the specificity of various phospholipases by direct product observation and should also be very useful in regiospecific chemical synthesis of phospholipids.

We found the ^{31}P-NMR chemical shift of monomeric dihexanoyl PC increases upon the addition of the nonionic detergent Triton X-100. This phenomenon was used to quantitate the solubilization of this phospholipid by the detergent micelles as a function of detergent concentration using a simple phase separation model (5). Similar studies were carried out on dibutyryl PC. At a phospholipid concentration of 7 mM and 56 mM detergent, 85% of the dihexanoyl PC, but only 3% of the dibutyryl PC was incorporated into the micelles.

Having shown that dibutyryl PC is monomeric under the enzyme assay conditions, we found that the phospholipase A_2, which acts poorly on PE in mixed micelles, is activated by dibutyryl PC which is itself an even poorer substrate. ^{31}P-NMR spectroscopy was employed to show that only PE is hydrolyzed in mixtures of various compositions of these two phospholipids. The fully activated enzyme hydrolyzes PE at a similar rate to its optimal substrate, PC containing long-chain fatty acid groups. Because dibutyryl PC is not incorporated into the micelles, these results are consistent with a mechanism of direct activation of the enzyme by phosphoryl-choline-containing lipids (either monomeric or micellar) rather than a change in the properties of the interface being responsible for the activation of phospholipase A_2. Therefore, two functional sites on the enzyme have to be assumed: an activator site and a catalytic site (6).

The determination of the positional specificity of phospholipases by the direct observation of product formation requires that the migration of the remaining fatty acid group in the lyso-phospholipid product be slow compared to the enzymatic hydrolysis reaction. Therefore a kinetic investigation of this migration was carried out. One enantiomer of each of the three possible isomeric lyso-phosphatidylcholines (1-acyl-*sn*-glycero-3-phosphorylcholine, 2-acyl-*sn*-glycero-3-phosphorylcholine and 3-acyl-*sn*-glycero-2-phosphorylcholine) were generated enzymatically. The assignment of the glycerol portion in the ^{1}H-NMR spectrum allowed an unequivocal identification of these compounds. The ^{31}P-NMR spectrum of each gave a distinct peak. From the assignments, the ^{31}P-NMR spectrum of the lyso products could also be used to show the specificity of phospholipase A_2 for the *sn*-2 position and of lipase for the *sn*-1 position directly. This eliminates the necessity of regiospecifically radio-labelled phospholipids, which are difficult to synthesize with absolute isomeric purity. ^{31}P-NMR also allowed us to demonstrate the absolute specificity of cobra venom phospholipase A_2 for the *sn*-2 position of monomeric dibutyryl PC. for which there were no specifically radio-labelled phospholipids available and they would be even more difficult to prepare because of the facile acyl migration. The monomeric phospholipids do not have the

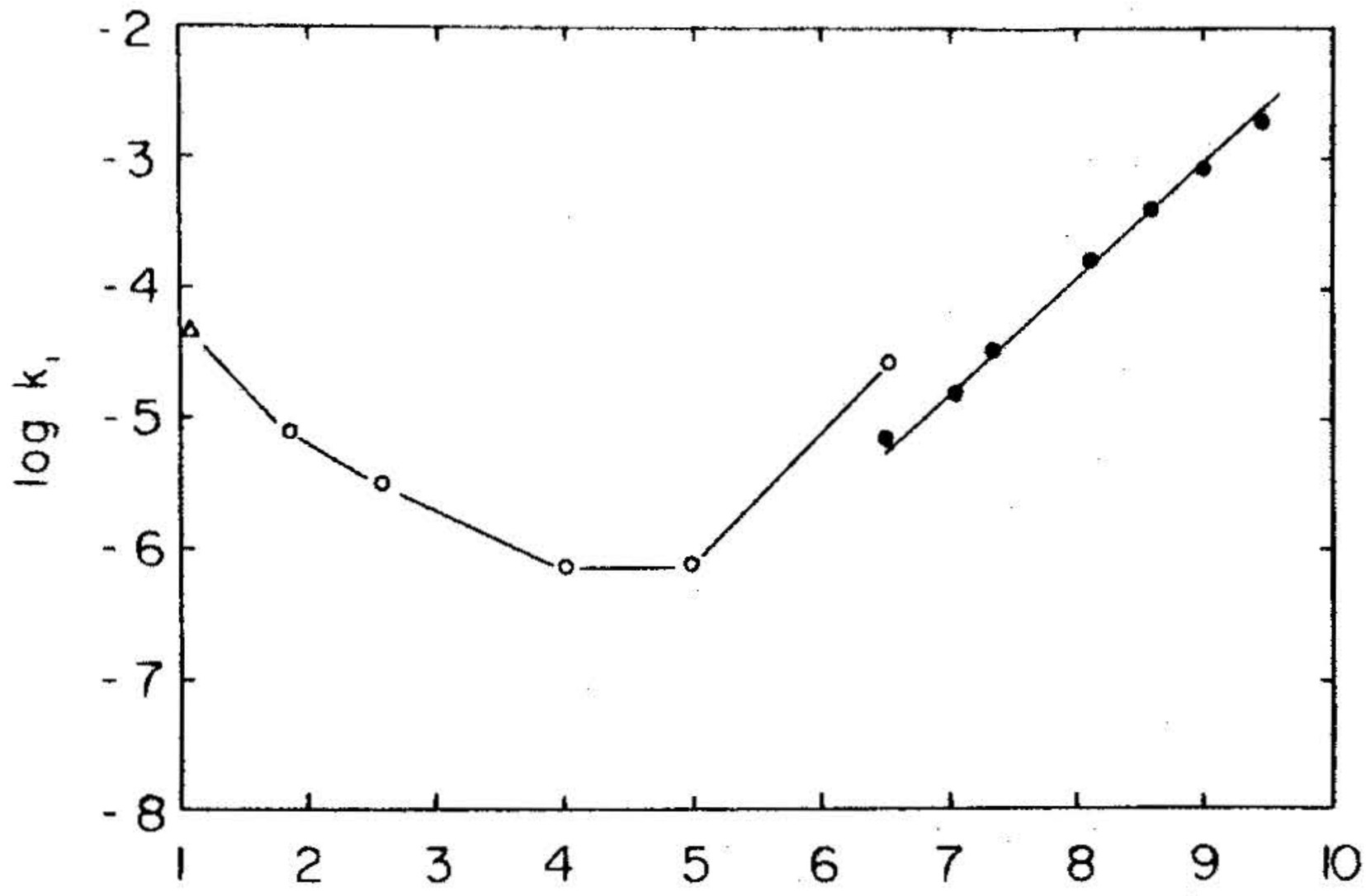


Figure 1: The pH-dependence of the acyl migration of lysophospholipids is shown. The log of the pseudo first-order rate constant k_1 *for the rearrangement of 2-palmitoyl-*sn*-glycero-3-phosphorylcholine into 1-palmitoyl-*sn*-glycero-3-phosphorylcholine is plotted against the pH buffered with 50m*M *tris HCl (●), 50m*M *citrate (○), or 0.1*M *HCl and 160m*M *Triton X-100 (△) to solubilize the reaction products at acidic pH.*

sn-2 carbonyl group preferentially exposed at the interface as do micellar phospholipids (7).

Although the isomeric lyso-phospholipids can in principle be distinguished by IR, ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR, only the latter provides the necessary sensitivity and well-resolved single resonance peaks that make it suitable as a kinetic tool to study the migration of the acyl-chain between the *sn*-1 and *sn*-2 position. The acyl migration was found to be both base catalyzed with a second order rate constant of $k_2 = 160\ M^{-1}\ s^{-1}$ and acid catalyzed, however much more slowly, with a second order rate constant of $k_2 = 4 \times 10\text{-}4\ M^{-1}\ s^{-1}$ (calculated between pH 1 and 2). The pH-rate profile is shown in Figure 1. At basic pH, the hydrolysis of the fatty acyl group is approximately 6500 times slower than migration. The equilibrium mixture in base contains approximately 90% of the 1-acyl isomer and 10% of the 2-acyl isomer. The phosphoryl migration was found to be too slow to measure, except at very acidic pH. Under these conditions, however, a variety of hydrolytic reactions also take place on a similar time scale so that the quantitation of the phosphoryl migration is complicated.

Two factors make the preparation of phospholipids that are specifically labelled in one acyl chain with a high degree of purity rather difficult: Acyl-migration during the preparation of the monoacyl intermediate and acyl migration during the reacylation in organic solvents. Basic catalysts that are used in common reacylation procedures of lyso-phospholipids also catalyze the latter migration. However, the possibility of directly observing it by ^{31}P-NMR provides a simple and convenient method to optimize conditions which minimize acyl migration. ^{31}P-NMR also allows one to test the isomeric purity of α- and β-phospholipids rapidly because the chemical shift of the β-phospholipid in mixed micelles is about 0.6 ppm upfield from the α-phospholipid. These compounds have been widely used in the study of model membranes.

1. Deems, R.A.; Eaton, B.R.; Dennis, E.A. J. Biol. Chem. 1975, 250, 9013.
2. Roberts, M.F.; Deems, R.A.; Dennis, E.A. Proc. Natl. Acad. Sci. U.S.A. 1977, 74, 1950.
3. Roberts, M.F.; Adamich, M.; Robson, R.J.; Dennis, E.A. Biochemistry 1979, 18, 3301.
4. Adamich, M.; Roberts, M.F.; Dennis, E.A. Biochemistry 1979, 18, 3308.
5. Plückthun, A.; Dennis, E.A. J. Phys. Chem. 1981, 85, 678.
6. Plückthun, A.; Dennis, E.A. Fed. Proc. 1981, 40, 1805.
7. Roberts, M.F.; Bothner-By, A.A.; Dennis, E.A. Biochemistry 1978, 17, 935.

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