Description of Module

Subject: Biochemistry

Paper: 03 Structure and Function of Biomolecules II
Module: 21 Phospholipids

Production of Courseware
- Content for Post Graduate Courses

Principal Investigator
Dr. Sunil Kumar Khare, Professor
Dept. of Chemistry,
I.I.T. Delhi

Paper Co-ordinator
Dr. Sunil Kumar Khare, Professor
Dept. of Chemistry,
I.I.T. Delhi

Content Writer:
Dr. M.N.Gupta, Emeritus Professor
Dept. of Biochemical Engg. and Biotechnology, I.I.T. Delhi

Content Reviewer:
Dr. Prashant Mishra, Professor
Dept. of Biochemical Engg. and Biotechnology, I.I.T. Delhi

Biochemistry
Structure and Function of Biomolecules II
Phospholipids
<table>
<thead>
<tr>
<th>Subject Name</th>
<th>Biochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paper Name</td>
<td>03 Structure and Function of biomolecules II</td>
</tr>
<tr>
<td>Module Name/Title</td>
<td>21 Phospholipids</td>
</tr>
</tbody>
</table>
Objectives

- To learn about the structure and importance of glycerophospholipids
- To learn about plasmalogens and their occurrence
- To learn about various phospholipases and their applications in biochemistry

Concept Map

```
Phospholipids
  ├── Degradation by phospholipases
  │    └── A₁
  ├── Importance
  │    └── A₂
  ├── Structures
  │    └── C
  │ └── D
```
3. Description

Apart from C, H, O, N; P is an important element in biochemistry. Nucleic acids are formed by nucleotides joined by the phosphodiester bond. As RNA world existed before the DNA world, the role of P in biological molecules during evolution happened early.

Biomembranes contain phospholipids. That also signifies the importance which evolution attached to exploiting phosphorus chemistry. Let us devote this module entirely to this important class of biological molecules.

In glycerophospholipids and some sphingolipids, the polar part of the molecule is attached to rest of the hydrophobic part by phosphodiester linkages. Such molecules are called phospholipids. Not all sphingolipids contain phosphate. These sphingolipids have sugar(s) instead forming the polar head. These are called glycolipids and will be discussed separately in the next module.

Phospholipases hydrolyse phospholipids at various bonds. The early phospholipases were identified in snake venoms. Phospholipase C and D for quite a while held limited interest. As gradually their wide ranging roles emerged, their enzymology became quite exciting.

Glycerophospholipids

Some basic structural information about glycerophospholipids (and other phospholipids) is already provided in the early introductory module. We will built upon those basics after now having acquired little more knowledge about the importance of fatty acid components in shaping properties of the lipids of which they are constituents.
The simplest glycerophospholipid are phosphatidic acids. One can view these as derivatives of glycerol-3-Phosphate with C-1 and C-2—OH of the glycerol-3-Phosphate esterified with fatty acid acyl chains. Acids, because of the presence of phosphate group forming a monoester with the terminal —CH₂-OH of the glycerol. The phosphate group is ionised and can pick up a H⁺.

![Glycerophospholipid](image)

**Choline**

![Serine](image)

**Ethanolamine**
Phosphatidic acids are present in biomembranes but in very small amounts. When the phosphate group forms a diester with another alcoholic group like that of choline, Ser or ethanolamine, the corresponding compounds named as phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine are formed. Phosphatidyl choline is also known as lecithin.

These glycerophospholipids occur commonly in bio-membranes. Generally, fatty acids at C-1 are saturated C\textsubscript{16} or C\textsubscript{18}. At C-2 fatty acids are often unsaturated fatty acids of C\textsubscript{16}-C\textsubscript{20} chain length. These amphiphiles have the two acyl chains as nonpolar tails with one polar head consisting of the phosphate esterified with the alcohol from choline/Ser/aminioethyl moiety.

The formula of one such glycerophospholipid 1-Stearoyl-2-oleoyl-3-phosphatidycholine is shown here. In the space filling model, please note the bent shape of the oleoyl chain as compared to the straight palmitoyl chain in the molecule. Little later, we will see the significance of this when we discuss the role of lung surfactants in the function of that organ.

The nature of the fatty acids present in any kind of phospholipid (like phosphatidyl choline) can vary widely. This variation can occur in the same cell or same tissue or within same organism. The distribution of the different molecular species also varies from organism to organisms.
Diphosphatidyl glycerols also occur. Its most important example is cardiolipin. Two phosphatidyl groups share the same glycerol in such molecules. Cardiolipin was first isolated from the heart tissue, hence so named. It is however also present in other tissues of the animals. This phospholipid is a good immunogen.

Phosphotidylcholine and phosphotidyl ethanolamine are derived from diacylglycerol. Phosphatidyl ethanolamine can be transformed to phosphotidyl serine in animals in an exchange reaction. The enzyme catalysing this reaction requires Ca\textsuperscript{2+}, situated on the cytosolic side of the endoplasmic reticulum, it is called base-exchange enzyme.

**Ether phospholipids**
Some analogs of phosphatidyl choline contain an ether linkage instead of acyl unit at C-1 carbon. These glyceryl ether phospholipids are fairly extensively distributed in animal tissues. While in most of the tissues, their overall % of the even total phospholipid content is not large, their concentration is much higher in erythrocytes and bone marrow. Interesting enough, in slugs and some molluscs, upto 25% of the phospholipid content can be these ether phospholipids. Their precursor is dihydroxyacetone phosphate, the glycolytic intermediate.
1-alkyl-2-acetyl ether derivative of phophatidyl choline is a platelet activating factor. At low concentration of 0.1nM, it is capable of aggregating blood platelets. It also causes dilation of blood vessels.

A simple substitution of long chain fatty acid by the acetyl group in this compound is enough to enhance the compatibility of this molecule to function in predominantly aqueous environment of the blood.

This platelet activating factor is released from basophils. As we discuss in the immunology section basophils are white blood cells which are stained with basic dyes. Upon being secreted by basophils, it also induces platelets to release serotonin.

This interesting phospholipid also affects in diverse ways other organs like liver, smooth muscle, uterus and lung. It is also involved in inflammation and allergic responses.

**Plasmalogens**

![Plasmalogens reaction](image)

Plasmalogens are also ether phospholipids. Their structural characteristic is that C1 has an α, β-unsaturated ether. The alkyl precursor undergoes loss of two hydrogen catalysed by a microsomal enzyme desaturase.

The reaction actually is similar to the one which takes place when double bond is introduced into saturated long chain fatty acids during their synthesis. The reaction requires NADH+O₂ and participation of cytbs₅.

Phosphatidal choline is a plasmogen corresponding to phosphatidyl choline. The ester group at C1 of the latter is replaced by the unsaturated ether. In the name, ‘y’ is replaced by ‘a’.

In vertebrates, nearly 50% of the heart phospholipids are plasmalogens. The other rich sources of ether phospholipids are membranes of halophilic bacteria, ciliated protists, and some invertebrates.
Plasmalogens are also synthesized from Dihydroxyacetone phosphate as a precursor. The most common bases present in plasmalogens are ethanolamine, choline and serine which are added to the structure from there CDP derivatives.

In a genetic disorder called zellweger syndrome, the liver and brain cells are deficient in plasmalogens. The infants born with this syndrome die early. It is believed that plasmalogens play an important role during embryonic development.

**Lecithin as a by-product in oleochemical industries**

Edible oils need to be refined before being sold as a premium product. When obtained in their crude from, many oils (to a varying extent) contains gummy material, its removal is called degumming.

In the classical industrial practice, oil is mixed with water and a degumming agent. Such degumming agents include citric acid, phosphoric acid, oxalic acid, acetic anhydride or even maleic anhydride. The mixture is continuously agitated. A lecithin sludge is obtained which consists of many phospholipids, phosphatidic acid and some glycolipids and even oligosaccharides. Depending upon the extent of removal of water content by evaporation during degumming, the lecithin sludge so obtained can be either a highly viscous material or a powder.

Industrially, soya bean has been the main source for obtaining lecithin. Some other sources like rapeseed, sunflower and corn have also been tried. Soybean remains the main source because of its functional superiority.

Neutral and polar lipids are extracted from the crude lecithin by acetone extraction. Use of ethanol or ethanol water co-solvent mixtures extracts phosphotidyl choline and phosphatidic acid + phosphotidyl inositol are mostly removed.

This product itself can be used as an oil-in-water emulsifier in food processing. It is used for margarine production. The higher ratio of phosphotidyl choline to phosphotidyl ethanolamine makes the preparation a better emulsifier.

The insoluble part contains acidic phospholipids and are used in water-in-oil system. Viscosity of chocolate mass is increased by adding this preparation and partly replaces the requirement of cocoa butter. So, industry producing chocolate has a requirement for this product which in itself is a by product of lecithin purification process.

Purified lecithin is a highly priced product and is obtained by adsorption chromatography with media like aluminium oxide, silica gel or DEAE-cellulose. Industries which use lecithin include pharma, cosmetics, and food. The main utility is in stabilizing water and oil emulsions as an emulsifier. The level of purity required varies with the application. The composition, especially presence of other
phospholipids and fatty acid content, dictates not only emulsifying quality but also whether oil-water or water-oil emulsion is to be stabilized.

Many cosmetics contain 0.5-1% lecithin which imparts “skin-feel” to these products. Ease of spreading, wetting ability, reduction of oily or greasy feeling are some of the useful results of incorporating lecithin in such products.

Industrial coatings, paints and inks contain lecithin as a dispersing agent for the pigment. Bakery, beverage and confectionery products use lecithin as a food grade emulsifier. In baking, it is used as a dough conditioner and aids mixing of fat, flour or sugar.
For many other applications, lecithin is modified chemically or by using enzymes like phospholipases. Hydroxylated lecithin, for example is a better oil-in-water emulsifier. Regulatory authorities prohibit use of most of the modified lecithin products in processed food.

Dipalmitoyl phosphatidyl choline (DPPC) as a component of lung surfactant
Lung surfactant is a mixture of protein and lipid of which 80-90% (w/w) is phospholipid. About 70-80% of these phospholipids is mostly DPPC alone.

What makes DPPC a useful constituent of lung surfactants? The saturated long chain of palmitoyl moieties extend without bends (as unsaturated bonds would have created). This allows the molecules to pack as a single layer.

The orientation is polar phosphatidyl choline head faces alveolar cells. These are the cells in the lung which form air spaces called alveoli. The surfactant coats the surfaces of these cells. The nonpolar layer of the palmitoyl chains face the air. During the breath expulsion, the alveolar volume and surface decrease, the layer hinders complete collapse of the alveolar space.
This also facilitates the expansion-contraction cycle of the alveolar cells. Expansion of a totally collapsed alveoli would be more inefficient in terms of effort required. Alveolar cells continuously synthesize and release the surfactant. Premature infants have very little production going on. Hence these babies are more prone to alveolar collapse. Such a situation leads to respiratory distress syndrome. In adults, an injury of the lungs can lead to insufficient surfactant production. The result is adult respiratory distress syndrome. In both cases, giving exogenous surfactant helps. The respiratory distress syndrome, as the name suggests is the difficulty in breathing. So, in the structure of DPPC, the critical design is absence of unsaturation in fatty acid chains.

**Phospholipases**

To the uninitiated, the nomenclature of phospholipases can sometime appear to be confusing. The best way out is to start with a recap of the early enzymology of these enzymes. Phospholipases are hydrolases. While lipases digest fats/oils, phospholipases digest some phospholipids. Liver and egg yolk were known to contain Lecithins. Around mid-1950s it was found that lecithin is digested in the gastrointestinal tract by an enzyme which was also present in the pancreatic juice. This enzyme was called lecithinase.

It was soon realized that since lecithinase is hydrolysing acyl ester bonds on the glycerol, there may be similar enzymes which will also hydrolyse phosphatidyl serine or phosphatidyl amino ethanol. Hence, it was proposed that instead of lecithinase, all such enzymes are called phospholipases.

Another enzyme which removes fatty acid from the middle C-atom of the glycerol skeleton in the lysolecithin (it give L-α-Glycerylphosphoryl-choline) was called lysolecithinase. Lecithinase and Lysolecithinase were called lecithinase A and Lysolecithinase B. Eventually, these were called phospholipase A and phospholipase B respectively.

It was known that lecithinase occurs not only in animals. It also occurs in plant tissues, microorganism, snake venom, bee venom and scorpion venom. The physiological action of the venoms was, for a long time, thought to be due to lecithinase or phospholipase A alone.

The actual picture is quite complex to be discussed here. These venoms also contain toxins. Some X-ray differaction work on the phospholipases and these peptides are slowly bringing clarity to the picture.

Lysolecithinase B was also known to occur in animals, plants and microorganisms. The substrate for this enzyme, various lysolecithins, in fact were prepared by using phospholipase A action on various lecithins.
Lysolecithinase of the mold *Penicillium notatum* was used to obtain L-α-glycerylphosphoryl choline by Uziel and Hanahan in 1956. The older literature mentions lecithinase B. As protein purification techniques became more powerful this preparation was actually found to contain both phospholipase A and lysolecithinase.

Even in 1940s, another phospholipase was known to occur in mammalian brains, snake venoms, and an anaerobic bacteria called clostridium welchii. C. Welchii was known to microbiologists as gas gangrene bacillus. This phospholipase hydrolysed phosphatidyl choline to phosphoryl choline and a diglyceride. It was given various names: Cl. Welchii lecithinase, Cl. Welchii α-toxin, glycerophosphates, lecithinase D. It was eventually called phospholipase C.

During the same period, a phospholipase was found in higher plants like cabbage, carrot and cotton seed which had different specificity towards lecithin and other similar phospholipids. This was found to produce α-phosphatidic acid and the base linked by the phosphodiester bond.

The enzyme at one time was called phosphatidase C but was later on termed as phospholipase D. Its early enzymology was carried out with both peanut and savoy cabbage. Phospholipase D turned out to be more widespread than just plants. Its assay was little cumbersome, so the enzymology did not take off fast. However, many other assays were developed once its importance in biochemistry become well established.

It is interesting to note that phospholipase C was reported to be active in ether-ethanol solution. This was decades before Klibanov at MIT described the catalysis of protease and lipases in organic solvents as the reaction media.
Specificity of phospholipases

Let us look at the current nomenclature of phospholipases.

The bonds which are hydrolyzed by various phospholipases are as shown in the figure. Phospholipases in general have different roles in biology (in vivo) and in biotechnology (in vitro).

In living animals, phospholipases are present in the intestinal juice and take part in the digestion of phospholipids. They have a similar role of digestive/hydrolytic enzymes in bacterial secretions and venoms. Phospholipases also are part of enzyme cascades and signal transduction.

Phospholipases A₁ and A₂ remove fatty acids by esterase action. Hence, these are unable to act on ether bonds present in ether phospholipids like plasmalogens. The product is a lysophospholipid. Some phospholipases are very specific e.g. one acting only on phosphatidyl choline, others are less specific.

The crystal structure of phospholipase A₂ from the rafflesnake crotalns atrox shows the enzyme to be a dimer. The subunits are identical. The pancreatic phospholipase A₂ was also found to have a similar structure.

The pancreatic enzyme however functions as a monomer. Produced as a zymogen, trypsin removes a haptapeptide from the N-terminal. The enzyme has a great stability. It retains its activity in the presence of 8M urea and substantial activity even in the presence of 2% SDS.

A protein lipocortin has been found to be an inhibitor of phospholipase A₂. A protein of molecular weight of around 37kD, it is induced by glucocorticoids. The inhibition seems to be via its interacting with the phospholipid substrate.

Lysophosphatidic acid (1-acyl-glycerol-3-phosphate) is produced by hydrolysis of membranes of blood platelets and cells which have been injured. It acts as a signal for initiation of healing/repair process.
Phospholipase C hydrolyses PIP$_2$ to generate PIP$_3$ and diacyl glycerol. Both molecules act as secondary messengers in signal transduction. This phosphoinositide pathway is discussed in more detail elsewhere.

Phospholipase D has been found in bacterial, plant, yeast and animal cells. Some phospholipases D are quite non-specific and will produce phosphatidic acid and the other alcohol. Even sphingomyelins, phosphatidyl glycerol and N-acylphosphatidyl-ethanolamine have been found to be substrates. Mammalian PLD1 and PLD2 are specific for phosphatidyl choline and have been cloned. Other mammalian PLDs specific for other phospholipids have also been detected in several tissues.

Phospholipases have proved very useful in modifying natural phospholipids to tailor their properties. Such phospholipids find considerable applications in plasma, food, cosmetic industries. Both drug delivery and gene transfer technologies have utilized phospholipids as vehicles.

In principle, phospholipases can catalyze both hydrolytic and reverse reactions. Both hydrolysis and transphosphotidylation of lyso phosphatidyl choline with PLD preparation from savoy cabbage was investigated.
For reverse reaction, 2M glycerol was used which helped in suppression of hydrolysis and reverse reaction. However unlike in hydrolytic reaction, detergents did not help the enzyme activity in the reverse direction. An example which further illustrates the synthetic utility of phospholipases was the synthesis of 1-lauryl-rac-glycerophosphate and 1-lauryl-dihydroxyacetone phosphate by combination of phospholipase C and phospholipase D. A two phase system of diethylether/H₂O facilitated the recovery of water insoluble products and hence was used as the reaction system.

Two important points are worth noting. First, phospholipids are just not constituents of the membranes. When released and hydrolyzed, they produce molecules which are involved in regulation of metabolism and signal transduction. Second, is the importance of phospholipids and phospholipases in industry. Lecithin is an established industrial grade emulsifiers acceptable in food and pharma and cosmetic industries. Phospholipases similarly are useful as reagents to engineer new lipids.

Summary

- Structure of Glycerophospholipids and their role
- Ether phospholipids including plasmalogens
- Lecithin as a by product in oleochemical industry
- Phospholipases and their biochemical importance