REVIEW ARTICLE

PHOSPHATIDYLETHANOLAMINE AND LYSOPHOSPHATIDYLETHANOLAMINE

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Phosphatidylethanolamine and Lysophosphatidylethanolamine belong to a group of phospholipids generally termed cephalins. The phospholipids are universally distributed among living organisms and are thought to be essential components of cell membranes. From the quantities of lipoidal and protein material found in cell membranes (Danielli and Stein, 1956) together with surface tension and related studies (Danielli and Harvey, 1935; Danielli and Davson, 1935), the membranes are considered to consist of a bimolecular leaflet of lipids with a unimolecular layer of protein adsorbed onto the polar surface.

The course of investigation of the cephalin fraction has not been reviewed in recent years, and here is discussed the general scope of the work in this field, under the headings of preparation, structure, synthesis and physical properties.

Preparation

Phosphatidylethanolamine

The cephalin fraction was first isolated and recognised as a separate entity apart from lecithins by Thudichum in 1884. He extracted it from brain (hence the name) and used its sparing solubility in warm ethanol as a method for separating it from lecithin.

Trier, in 1912, made the significant observation that when phospholipids were treated with cadmium chloride the lecithins formed a relatively insoluble complex whilst the cephalins formed a soluble complex in ether. Maclean (1915) utilised this knowledge and obtained a greatly improved separation of lecithins and cephalin.

Obata, Nukata and Sasa (1950) in attempting to extract the cephalin fraction from soybean, showed that the cadmium chloride method was not applicable to plant material. It was necessary to treat an emulsion of the soybean phospholipids with lead acetate and to centrifuge and boil the precipitate with methanol, before adding the cadmium chloride. The cephalin was released from the complex by adding hydrochloric acid.

Kirk (1938) introduced a method of separation in which the phospholipid mixture was first precipitated with acetone and magnesium chloride, and then extracted with moist ether. Kirk claimed that moist ether dissolved lecithins and cephalins but not sphingomyelin. This method was criticised by Sinclair and Dolan (1942) who indicated that the residue left from the extraction with moist ether was not primarily sphingomyelin, but merely a portion of the mixture of phospholipids, since the amount precipitated was proportional to the concentration of magnesium chloride added with the acetone.

The Reinecke salt was suggested by Thannhauser and Setz (1936) as a specific precipitating agent for sphingomyelin. Despite claims of complete precipitation of the sphingomyelin, the evidence did not preclude the possibility of other phospholipids being precipitated.

Folch and Schneider, in 1941, showed that cephalin was not a single substance, as the methods of preparation yielded substances having not all of the base as ethanolamine, some 40–70 per cent being serine.

Later, Folch and Woolley (1942) demonstrated the existence of a phospholipid in the cephalin fraction containing inositol.

Folch (1942a) then proved the existence of three fractions in the cephalin portion. By adding successive quantities of ethanol to a solution of cephalin in chloroform, precipitates of inositol-, serine-, and (by the addition of acetone) ethanolamine-containing fractions respectively were obtained. The significant point was that phosphatidylethanolamine, unlike the cephalin portion as a whole, was freely soluble in ethanol.

A similar separation was obtained by Lovern (1949), but he found that solvent fractionation still gave compounds that were far from pure, and suggested that chromatographic procedures would give better separations.

Chromatographic Separations

Adsorption chromatography was applied to the separation of mixed lipids by Trappe in 1940. Using magnesia as adsorbent, he showed that lipids were more strongly adsorbed as their polarity increased.

Other workers (Taurog, Entenmann, Fries and Chaikoff, 1944; Fauré, 1950; Hanahan, Turner and Jayko, 1951) used magnesia and alumina as adsorbents and successfully separated choline-containing from amino-containing lipids. However, they did not recover the cephalin fraction.

Rhodes (1956) obtained a separation and recovery of lecithin and cephalin fractions of egg yolk phospholipids by chromatography on alumina. By eluting with a solvent of methanol-chloroform (1 : 1 v/v) the lecithin travelled as a sharp band with the solvent front, and on changing to a solvent of ethanol-chloroform-water (5 : 2 : 2 v/v) the cephalins were eluted in 92–98 per cent yield. He suggested that by the use of less powerful eluents at the second stage, fractionation of the cephalins would be obtained.

Paper chromatography was first used to separate phospholipids by Chargaff, Levine and Green (1948). They worked with minute quantities and after hydrolysing the lipids by boiling with hydrochloric acid, they identified the liberated bases by paper chromatography. Choline was detected by reaction with phosphomolybdic acid, followed by reduction to molybdenum blue with stannous chloride. Amino-containing bases were detected by staining with ninhydrin. They later (1951) used these staining techniques for quantitative determination of the bases.

Hecht and Mink (1952) employed a filter paper method described earlier for the separation of amino-acids (Consden, Gordon and Martin, 1944). This involved the successive use of various solvents (phenol: water, phenol : ammonia, butanol : water, butanol : pyridine and butyric acid : water) and a fractionation of the cephalins was obtained. Since

the various products all gave the same ninhydrin-reacting bases on hydrolysis, it is difficult to interpret their results.

In 1951 Bevan, Gregory, Malkin and Poole claimed that it was possible to separate choline-containing phospholipids from those containing serine and ethanolamine using filter paper or cellulose columns. This was apparently confirmed by Hack (1953) who stated that amino-containing phospholipids were firmly bound to cellulose, whilst those containing choline ran with the solvent front. Lea and Rhodes (1953) were unable to confirm this separation. They noted that amino-acids were strongly bound to cellulose, while the phospholipids moved together in a solvent of chloroform : ethanol : water (800 : 200 : 25 v/v). This was confirmed in the subsequent removal of eleven free amino-acids from the column.

The cellulose method, therefore, offers a convenient means of removing amino-acids from lipid mixtures.

The use of silicic acid as an adsorbent for chromatography of lipids was first made by Kauffman in 1939, and in the following year by Trappe. In 1944, Rathman found that silicic acid was to be preferred to other adsorbents for column chromatography of phospholipids.

Diemair and Poetsch in 1949 extracted the phospholipids from yeast, and separated them by adsorption on silica gel. They stated that the various lipids could be recognised by their luminescence under ultraviolet light. This was criticised by Lea, Rhodes and Stoll (1955) who suggested that the fluorescence was contributed by deterioration products.

Fillerup and Mead (1953) defined the conditions of column preparation, loading, diameter and length, for the separation of lipid mixtures. By employing solvents of different polarity, fractionation of the main classes of lipids into their constituents was obtained.

Lea and Rhodes (1954) obtained a satisfactory separation of cephalins from lecithins of egg yolk (amino-acids having previously been removed with cellulose) on silica impregnated papers, prepared by the method of Kirchner and Keller (1950). Using a solvent of methanol-chloroform (1 : 3 v/v), the amino-containing phospholipids were fast moving, while the choline-containing substances were slower. In later publications (Lea, Rhodes and Stoll, 1955; Lea and Rhodes, 1955) a full list of R_F values was given and these values, which were observed to alter slightly from paper to paper, were subsequently shown to be due to variation in the water content of the paper. They also prepared columns of commercial sodium silicate which had been previously washed with 10N hydrochloric acid to remove iron and aluminium impurities, and filtered to remove the finely divided material that blocked the column. After drying, the silicic acid was "activated" by heating overnight at 110° (Mallinkrodt AR silicic acid worked well with or without activation). This was slurried with a solvent of methanol-chloroform (1 : 3 v/v), poured into the column and allowed to pack under a slight pressure of nitrogen. A similar separation was obtained to that with the impregnated paper, except that lysophosphatidylethanolamine could not be separated from lecithin on the column.

Lea (1955) continued this work and found no difficulty in obtaining phosphatidylethanolamine free from choline-containing lipids and containing only traces of substances with amino-acid nitrogen. Running just ahead of the phosphatidylethanolamine was a group of yellow-brown pigments which required care to separate cleanly without the use of a second column. In view of the tendency of unsaturated fatty material to autoxidise when adsorbed on to solid surfaces, the silicic acid was heated to 100° under high vacuum to remove adsorbed oxygen, and then nitrogen was admitted. De-aerated solvents were used, and in one case, Lea even passed a previously prepared sample of phosphatidylethanolamine down the column to adsorb any remaining oxygen, before making a separation on the column, but results showed that this procedure was unnecessary.

Hanahan, Dittmer and Warashina (1957) used columns of silicic acid to separate the mixed phospholipids from rat liver, beef liver and yeast. By varying the solvent proportion (chloroform-methanol, 4:1, then 3:2, then 1:4 v/v) a satisfactory separation of phosphatidylethanolamine, phosphatidylserine, phosphoinositides and lecithins was obtained.

Hirsch and Ahrens in 1958 gave meticulous details for the pre-treatment of silicic acid before its use for phospholipid separations. Since some workers had found that separations could be unreliable, a systematic study of the variables was reported; these were, particle size of adsorbent, adsorption values, water content, column preparation, loading capacities and solvents. They indicated that abrupt changes of solvent gave sharper separations of the phospholipids than was obtainable by gradient elution, thus confirming studies by Hanahan and others (1957). In direct contradiction was a report by Wren (1959) who stated that, in dealing with lipid extracts, the need was for a continuous gradient elution. He described a simple apparatus for obtaining this with chloroform-methanol mixtures, and by reference to blood lipids, indicated that peaks were sharper, and less trailing than with discontinuous gradient elution. This view was supported later by Bader and Morgan (1962).

Different solvent systems were employed by other workers: Marinetti and Stotz (1956), Marinetti, Erbland and Kochen (1957) used di-isobutylketone: acetic acid: water (40:20:3 v/v) successfully with one and two dimensional silica-impregnated papers. Rapport and Alonzo (1955) used 30 per cent ethanol in hexane to elute amino-containing lipids from a silicic acid column, whereas 40 per cent methanol in hexane was required to elute lecithin. Dieckert and Reiser (1955) used methanol: ether (1:1 v/v) with an adsorbent of glass fibre impregnated with silicic acid, and obtained satisfactory separation of tissue phospholipids. Similar use of glass paper was reported by Agranoff, Bradley and Brady (1958), and Muldrey, Miller and Hamilton (1959).

Thin-layer chromatography was used by Wagner (1960) employing silica-gel suspensions as adsorbent. A good separation was obtained of a synthetic mixture of lecithin, lysolecithin, sphingomyelin, phosphatidylethanolamine, cerebroside and cardiolipin, using chloroform: methanol:

water (65 : 25 : 4 v/v) as a solvent. The advantages of thin layer chromatography were (*a*) rapidity, (*b*) good separations, and (*c*) emulation of conditions of column chromatography more realistically than with paper-chromatography. Wagner (1961) was able to determine the concentrations of phosphatidylethanolamine and lecithin in extracts of soybean oil, egg yolk, heart muscle and brain.

Jatzkewitz and Mehl (1960) determined the R_F values of over 30 test substances from brain lipids, by thin-layer chromatography, using eleven different solvents, with clean separations of phospholipids.

Vogel, Doizaki and Zieve (1962) used Mallinkrodt silicic acid with plaster of Paris as the binding agent for thin-layer chromatography of serum phospholipids, and obtained a clean separation of phosphatidylethanolamine, lecithin, sphingomyelin and lysolecithin. Cornatzer, Sandstrom and Reiter (1962), and Skidmore and Entenmann (1962) obtained good separations of mixtures of naturally occuring phospholipids.

Bungenberg de Jong and Hoogeveen (1960) described the use of silicon tetrafluoride for impregnating papers. Using a solvent of di-isobutylketone : acetic acid : water (50 : 25 : 5 v/v) a separation of phospholipids was obtained, and in a later report Bungenburg de Jong (1961) gave details of the mechanism of the separation of phosphatidylethanolamine and lecithin on such papers.

Horhammer, Wagner and Richter (1959) were able to obtain reproducible separations of phospholipids by using specially prepared formaldehyde papers, and a mixture of butanol, acetic acid, and water as the solvent. They also reported the use of Malachite Green as a specific reagent for detecting lysophospholipids.

Mumma and Benson (1961) reported the use of anion exchange papers in the separation of lipid mixtures. Reproducibility, freedom from oxidative adsorption and commercial availability of the papers were the advantages. However, the order of the R_F values of phosphatidylethanolamine and lecithin were reversed and were rather too close together for general preparative work.

Counter-current Separations

Scholfield, Dutton, Tanner and Cowan (1948) and Scholfield, Dutton and McGuire (1950) were the first to apply this technique to phospholipids, using a metal apparatus of the same design as Craig's (1944). Later Kies and Davis (1951) extended the method to involve a series of liquid-liquid extractions in which one phase continually flows over the other, termed the "cascade" technique. This greatly extended the scope of the method and was used by Lovern (1952) to obtain a useful degree of separation of lecithin from amino-containing lipids from ox brain. The separation of phosphatidylethanolamine from phosphatidylserine was poor.

Cole, Lathe and Ruthven (1953) employed a solvent system of water : methanol : carbon tetrachloride to overcome the difficulty of emulsification

during counter-current distribution. Working with fractions from brain lipids, they obtained a good separation of lecithin but, like Lovern, they found that phosphatidylethanolamine and phosphatidylserine were inseparable.

Scholfield and Dutton (1954) have claimed that after the precipitation of inositol-containing substances by the addition of lead acetate, a solvent system of hexanol-methanol gave phosphatidylethanolamine in 90–95 per cent purity.

However, Rhodes (1956) commented that as the solubility of phospholipids is so influenced by the constituent fatty acids, and coupled with the emulsification difficulties, the use of counter-current distribution may well be precluded.

Ion Exchange

Lea, Rhodes and Stoll (1955) considered the possibility of separating lecithin from phosphatidylethanolamine on ion-exchange resins, since the former substance is approximately neutral, whilst the latter is acidic. Using a strong cationic resin, and a solvent of water : ethanol : ether (5 : 3 : 2 v/v) or 95 per cent ethanol, lecithin was readily separated from phosphatidylethanolamine, but the capacity of the resin for the relatively large phospholipid molecule was so small (1–3 per cent) as to render the method useless for preparative work. Furthermore much hydrolytic decomposition took place.

Perrin and Sanders (1960) used Dowex ion-exchange resins in the bicarbonate form, and successfully removed the amino-containing lipids in mixed egg phosphatides in the preparation of lecithins. No recovery of the phosphatidylethanolamine was made.

Paper Electrophoresis

Employing a solvent of methoxyethanol : tetrachlorethane : water (47 : 47 : 6 v/v), Garvin (1956) obtained a satisfactory separation of lecithin, phosphatidylethanolamine and phosphatidylserine in a synthetic mixture. In subsequent work (1958) Garvin and Wallach gave the rates of migration on paper under precise conditions.

Zipper and Glantz (1958) studied the effects of various solvent systems, voltages and time periods. They confirmed that a satisfactory separation of lecithin, phosphatidylethanolamine, phosphatidylserine and diphosphoinositide could be obtained.

Lysophosphatidylethanolamine

Delezenne and Fourneau in 1914, prepared lysocephalin by treating an emulsion of egg yolk with cobra venom for three days. The acetone insoluble material was fractionated into lysolecithin and lysocephalin by crystallisation from ethanol.

Levene and Rolf (1923) added cobra venom to a suspension of egg yolk in phosphate buffer at 40°. The lysophospholipids were precipitated with cadmium chloride and the cadmium salts were decomposed and the liquid concentrated until a precipitate began to form which contained 75 per

cent lysocephalin. Since the lysocephalin was almost insoluble in chloroform compared with lysolecithin, it was purified by dissolving in chloroform and re-precipitating with ether. The product was purer than previously prepared.

King and Dolan, in 1933, used a similar method, but separated the mixture by fractional precipitation from glacial acetic acid, through the addition of ether and acetone. The lysocephalin was concentrated in the first fractions. These early methods in aqueous media undoubtedly resulted in poor yields and impure products.

In 1939, Chargaff and Cohen initially separated lecithin and cephalin by way of their cadmium chloride complexes but found it impossible to prepare lysocephalin by the action of snake venom upon the isolated cephalin fraction. However it had been reported (Van Leeuwen and Szent-Gyorgi, 1923; Dunn, 1934) that snake venom acted upon isolated cephalin to give lysocephalin, but was much less reactive than with lecithin.

This difficulty was encountered by Lea and others (1955) who were able to obtain a satisfactory preparation of lysolecithin when reacting venom with egg lecithin, but could not repeat this for phosphatidylethanolamine. They therefore prepared a lysolecithin-lysophosphatidylethanolamine mixture by the direct action of venom on egg yolk, and obtained the individual lyso compounds by chromatographic separation on silicic acid. Using a solvent of chloroform:methanol (80:20 v/v) the lysophosphatidylethanolamine was well separated from the lysolecithin.

Davidson, Long and Penny (1955) noted a lag period in the reaction of venom on lecithin in the presence of phosphatidylethanolamine, and concluded that phosphatidylethanolamine has a higher affinity for the enzyme, but is degraded more slowly.

Long and Penny (1957) showed that phospholipase A hydrolyses phosphatidylethanolamine more slowly than lecithin, requiring 24 hr. compared with 1–2 hr. for lecithin. However, the addition of a small amount of ammonia accelerated the reaction, degradation being complete in 6 hr.

De Haas and van Deenen (1961a) showed that nearly one molar equivalent of fatty acid was liberated from synthetic phosphatidylethanolamines by reacting with venom for 20 hr. at 30°.

STRUCTURE

Phosphatidylethanolamine

Thudichum (1884) was the first to investigate the structure of cephalin, and from hydrolysis studies he proposed two structures; one where the fatty acid and glycerol radicals were attached to the phosphorus by substitution of the hydroxyl groups in phosphoric acid, and the other was analagous to that already indicated for lecithin:

CH_2—fatty acid
|
CH—fatty acid
|
CH_2—phosphoric acid-base.

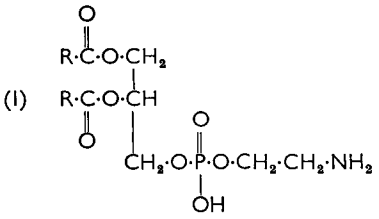
This second structure was found to be more correct after the finding of glycerophosphoric acid in the hydrolysis products (Frankel and Dimitz, 1909). The acid was optically active indicating the α rather than the β form. It had been shown by Bailly and Gaumé (1934) and Chargaff (1942) that chemical hydrolysis of methyl glycerophosphate caused the phosphoric acid moiety to migrate reversibly from the α to the β position, and an equilibrium mixture was obtained, the proportions of which depended on the pH. Folch (1942b) indicated the significance of this in analytical procedures involving hydrolysis of cephalins. Later comparative work with synthetic compounds (Baer, 1956; Long and Maguire, 1953; Baer, Stancer and Korman, 1953; Thannhauser, Boncoddo and Schmidt, 1951) indicated that natural phospholipids occured in the L-α-form.

Much controversy arose over the nature of the base in cephalin. Parnas (1909), Baumann (1913), and Renall (1913) showed that ethanolamine was the base, and found no evidence of any other nitrogen containing base, and that all the nitrogen could be estimated by the method of Van Slyke (1912). MacArthur (1914) showed that ethanolamine did not account for all the nitrogen, and from the large proportion of amino-acid present, he suggested that two cephalins existed; one having nitrogen in the ethanolamine form, and the other in the amino-acid form. This was later confirmed (Thierfelder and Schulze, 1915). Various suggestions came forward (MacLean and MacLean, 1927; Gray, 1940) to explain the divergence between the expected calculated elemental percentages and the observed results, but were not entirely satisfactory.

MacArthur, Norbury and Karr (1917) observed that the ethanol-soluble fraction of phospholipids contained its nitrogen equally divided between choline and ethanolamine, showing that the separation of lecithin from cephalin on ethanol solubility was entirely unsatisfactory. It was not until 1930, that Rudy and Page isolated from the ethanol-soluble fraction, a substance containing ethanolamine, which gave the correct analytical figures for the structure that had been ascribed to cephalin.

Folch (1942a) suggested that the ethanolamine ester of diacylglycerophosphoric acid is actually ethanol-soluble, and is not the material generally termed cephalin, which is characterised by its ethanol-insolubility. He proposed that this material be termed "Phosphatidylethanolamine" rather than cephalin. Together with Schneider (1941) he also isolated cephalin from the ethanol-insoluble fraction, and found it reacted with ninhydrin to give carbon dioxide in a manner similar to α-amino-acids. Analyses of various samples of the ethanol-insoluble fraction indicated that 40–70 per cent of the nitrogen was present in the amino-acid form, and it was suggested that the amino-acid was serine. Thus phosphatidylserine was isolated and explained the original analytical anomaly.

This lead to the assigning of the structure (I) to L-α-phosphatidyl-ethanolamine



where R represents the fatty acids.

Positionally, the fatty acids of phosphatidylethanolamine were assumed to be identical with those of lecithin. It was known for some time that the fatty acids split off from lecithin by phospholipase A were mainly unsaturated, and it had been shown (Hanahan, 1954a; Long and Penny, 1954) that these acids were removed from the α-position. Furthermore, Hanahan (1954b) claimed that the fatty acids are not distributed randomly in lecithin, but that the α-position is always occupied by the unsaturated, and the β-position by the saturated fatty acids, since reaction with phospholipase A removed all the unsaturation from the molecule.

However, Lea and Rhodes (1956) suggested that the situation is more complex than that found by Hanahan, since a small proportion (0·15 double bond per molecule) of unsaturation remains in the lyso compound of both lecithin and phosphatidylethanolamine indicating the presence of some unsaturated fatty acids in the β- as well as in the α-position. After hydrogenation, the released acids were found to be 47 per cent stearic and 53 per cent palmitic acids, proving the presence of C_{18} and C_{16} unsaturated acids in the original molecule.

Hawke (1959) found marked differences in the fatty acid composition of lecithin and phosphatidylethanolamine from egg yolk. Of the total fatty acids in phosphatidylethanolamine, 39·5 per cent were saturated C_{18} and 20·1 per cent unsaturated C_{18} acids, whereas in lecithin they were 14·2 and 44·7 per cent respectively. About 60 per cent of the total acids of phosphatidylethanolamine were saturated. His work showed that most of the acids attached to the α-carbon atom were unsaturated, whilst most of those attached to the β carbon atom were saturated.

Subsequent studies, however (Hanahan, Brockerhoff and Barron, 1960; de Haas and Van Deenen, 1961a), have shown that the distribution of unsaturated and saturated acids is the reverse of this, since phospholipase A liberates the fatty acids from the β-position.

Lysophosphatidylethanolamine

Lysophosphatidylethanolamine is obtained by the action of the enzyme phospholipase A upon phosphatidylethanolamine (Levene and Rolf, 1923; Fairbairn, 1945; Long and Penny 1957; de Haas and Van Deenen, 1961a; Robins and Thomas, 1963b). Some controversy has arisen over the position of attack of the enzyme. Ludecke (1906) found that the fatty acids released after enzymatic degradation of lecithin were invariably unsaturated, whereas the acid remaining was saturated. Despite a criticism of this by Latzer (1927) the finding was confirmed by Delezenne

and Fourneau (1914) and Belfanti, Contardi and Ercoli (1936), and this led to the thought that the enzyme specifically released the unsaturated acids.

However, Zeller (1925) found that the enzyme would attack a sample of L-α-dimyristoyl lecithin. Furthermore, King (1934) showed that hydrolecithin prepared by catalytic reduction of natural lecithin was attacked as rapidly as the original compound. This clearly indicated that the ester groupings of the unsaturated acids were not exclusively hydrolysed.

Chargaff and Cohen (1939) investigated the possibility that the enzyme preferentially attacked the more highly unsaturated acids, but found that this was not so.

Zeller (1925) postulated that the enzyme exclusively attacked at either the α- or β-position, irrespective of the state of unsaturation of the fatty acids at that ester linkage.

Hanahan (1954a) oxidised lysolecithin and subsequent acid hydrolysis yielded phosphoglyceric acid as the only phosphorus containing compound, indicating that the enzyme had attacked at the α-position. This was confirmed for lecithin and phosphatidylethanolamine in a similar investigation by Gray (1958).

Long and Penny (1954) worked on the hypothesis that if the fatty acid split off was from the α-position, then a free primary alcoholic grouping would be exposed: if from the β-position, then a secondary alcohol would result. From the quantity of potassium dichromate required to oxidise lysolecithin, it appeared that a primary alcoholic grouping had been exposed, and thus it was confirmed that the enzyme had liberated the fatty acid from the α-position.

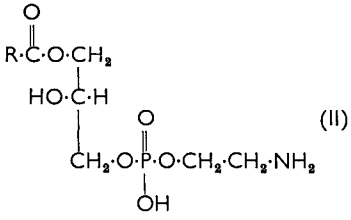
This was criticised by Marinetti, Erbland and Stotz (1959) who concluded from their results that the enzyme attacked indiscriminately at the α- or β-position. They later (1960) indicated that phospholipase A showed chain length specificity with respect to the hydrolysis of the fatty acids of lecithin, the C_{22} and C_{20} acids being hydrolysed in preference to the C_{18} and C_{16} acids.

Tattrie (1961), in investigating the positional distribution of fatty acids in lecithin, employed pancreatic lipase as a specific liberator of the fatty acids in the α-position. He showed that the saturated acids occupied the α-position and the unsaturated β-position. Since phospholipase A liberates the unsaturated fatty acids, then the site of attack must be at the β-position.

Hanahan and others (1960) re-investigated the problem and were able to confirm Tattrie's findings.

The difficulty has been conclusively settled for both lecithin and phosphatidylethanolamine by de Haas and van Deenen (1961a). They reacted phospholipase A with synthetic α-stearoyl β-oleoyl phosphatidylethanolamine and α-oleoyl β-stearoyl phosphatidylethanolamine and the former yielded oleic acid only, and the latter stearic acid only after enzymatic hydrolysis, thus proving that the enzyme has β-ester specificity. In view of these findings they suggested that the name "β-phosphatidase" be given to the enzyme.

Hence the structure of lysophosphatidylethanolamine must be (II).



(II)

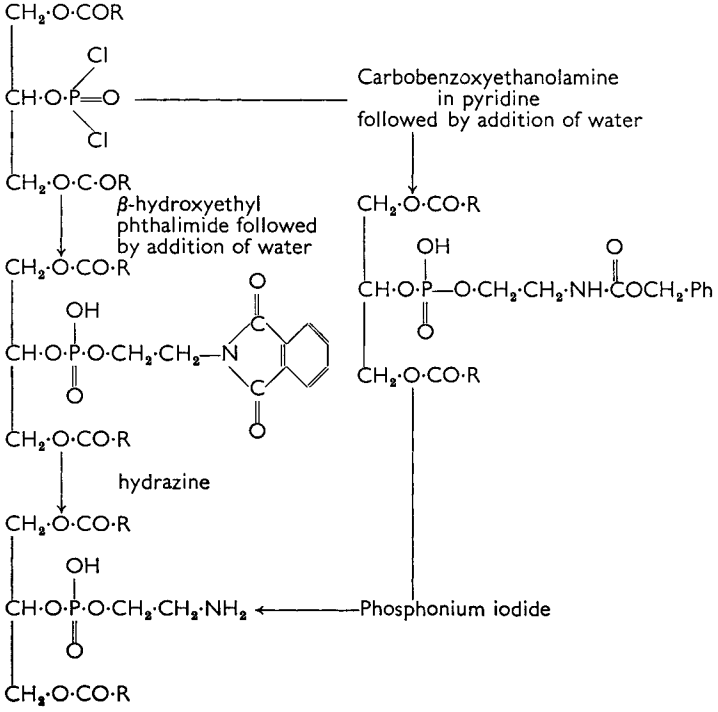
SYNTHESIS

Phosphatidylethanolamine

The first reference to an attempt to synthesise phosphatidylethanolamine was by Grün and Limpacher in 1927. They heated an $\alpha\beta$-diglyceride with phosphorous pentoxide and reacted this with the bicarbonate of amino-ethanol. It is very doubtful whether they obtained phosphatidylethanolamine, since the $\alpha\beta$-diglyceride had probably rearranged to the more stable $\alpha\gamma$-form, and the bicarbonate could not have completely prevented the phosphoric acid group from forming a salt with the amino-group of the ethanolamine.

Kabashima (1938) prepared an α-phosphatidylethanolamine by reacting an acetal of glycerol with phosphorous oxychloride, then stearoyl chloride, then introducing the ethanolamine moiety with bromoethylamine picrate.

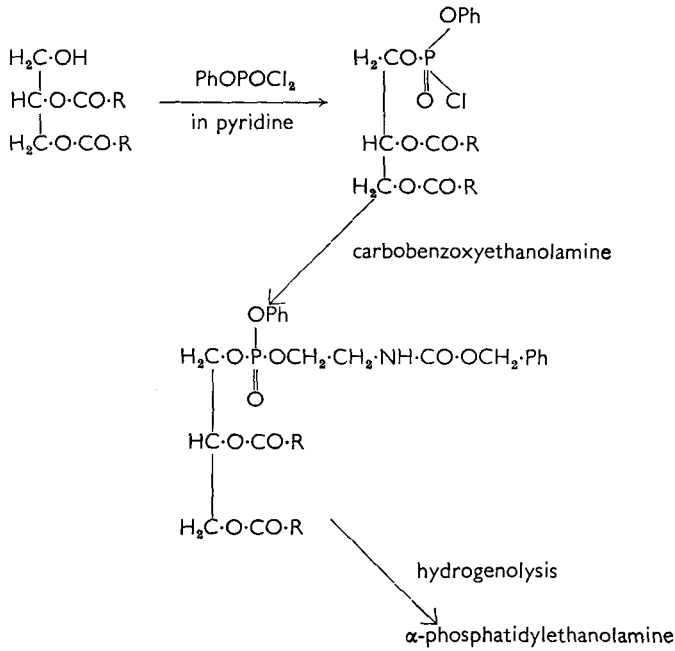
Rose (1947) commenced with an $\alpha\gamma$-dipalmitoyl ester of glycerol and by two routes obtained β-phosphatidylethanolamine.



This product was insoluble in ether, soluble in ethanol, sintered at 180° and melted at 194°. By a similar method, Hunter, Roberts and Kester (1948) prepared phosphatidylethanolamine containing two myristic acid groups, which had a melting-point of 174°. They did not isolate the intermediate phthalimide derivative, thus avoiding emulsification and crystallisation losses, so that the yield was greatly increased.

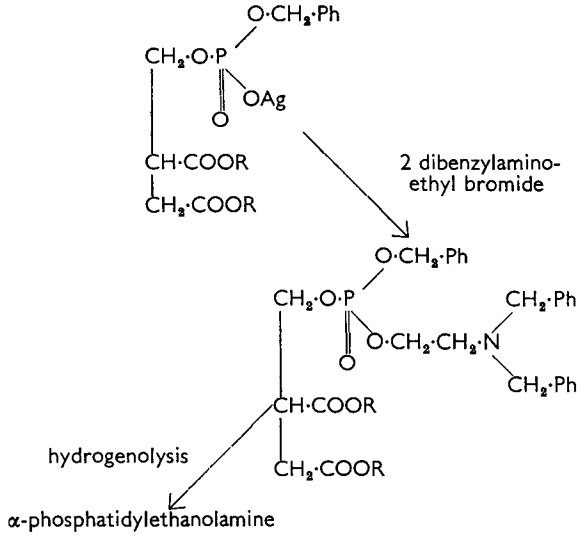
Baer, Maurukas and Russell, in 1951, claimed that no previous attempt to synthesize α-phosphatidylethanolamine could be considered successful, and reported the synthesis of three enantiomorphic compounds, the distearoyl, dipalmitoyl, and dimyristoyl L-α-phosphatidylethanolamines.

They commenced with a diglyceride which was phosphorylated with phenylphosphoryl chloride and then esterified with carbobenzoxyethanolamine in pyridine and then the protective groups removed by hydrogenolysis:



The products were insoluble in acetone and ether, but soluble in ethanol, benzene, pyridine and chloroform.

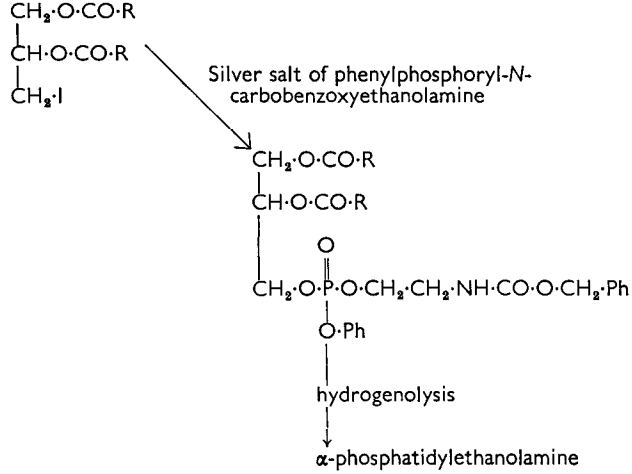
Hoefnagel, Stegerhoek and Verkade (1960) published a method of synthesizing the same α-phosphatidylethanolamines, by commencing with a substituted phosphate derivative of a diglyceride:



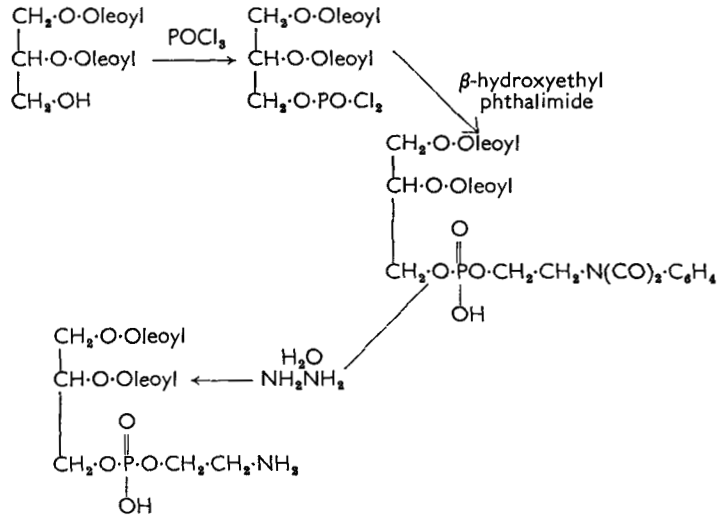
Baylis, Bevan and Malkin (1955) pointed out the difficulties that beset the routes of synthesis.

(1) Syntheses commence with $\alpha\beta$-diglycerides, which readily transform to the $\alpha\gamma$-form. (2) The action of reagents such as phenylphosphoryl dichloride on diglycerides tend to give rise to mixtures of esters which are difficult to separate. (3) To obtain unsaturated acids in the final product, it is necessary to prepare unsaturated $\alpha\beta$-diglycerides and to remove the protecting groups by some other method than catalytic hydrogenation.

They were able to overcome the first two difficulties by commencing with the appropriate iodohydrin:



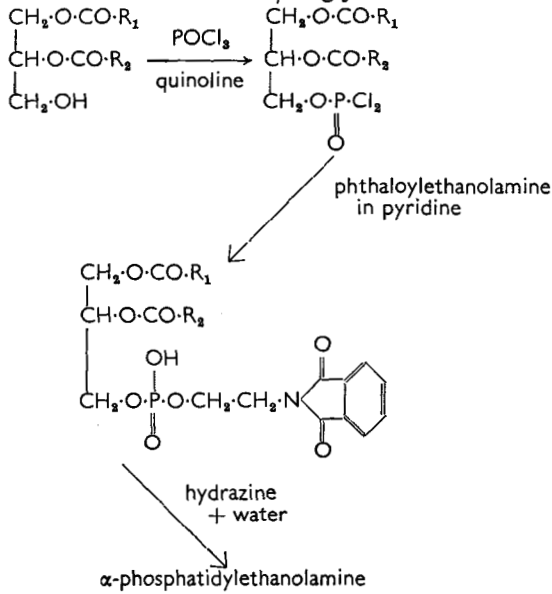
Later Baer and Buchnea (1959) were able to overcome the third difficulty and prepared dioleoyl phosphatidylethanolamine commencing with a 1 : 2-diolein:



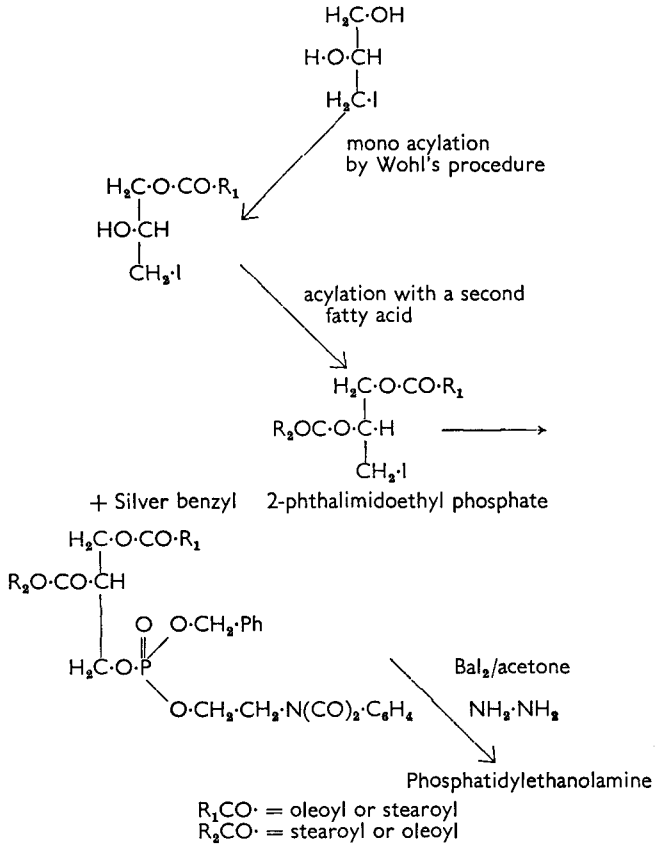
Bevan and Counsell (1961) synthesized DL-dioleoyl phosphatidylethanolamine by a method involving the use of an iodohydrin rather than the somewhat inaccessible 1,2-diolein.

Since, naturally occurring phospholipids appear to possess at least two dissimilar fatty acid substituents, one of which is unsaturated, this led various workers to attempt to prepare phosphatidylethanolamine containing one saturated and one unsaturated fatty acid.

Baer and Buchnea (1961) prepared α-stearoyl-β-oleoyl- and α-oleoyl-β-stearoyl-phosphatidylethanolamines by commencing with the correct enantiomorphic form of a mixed acid αβ-diglyceride.



De Haas, van Deenen and Daemen (1962) commenced with an iodohydrin and substituted the required fatty acids in separate steps:



Kennedy and Weiss (1956) demonstrated the function of cytidine coenzymes in the biosynthesis of phospholipids, by following the incorporation of radioactive phosphorus into the lipids. Two steps were involved, the first being catalysed by a cytidyl transferase enzyme, and the second by a glyceride transferase enzyme:

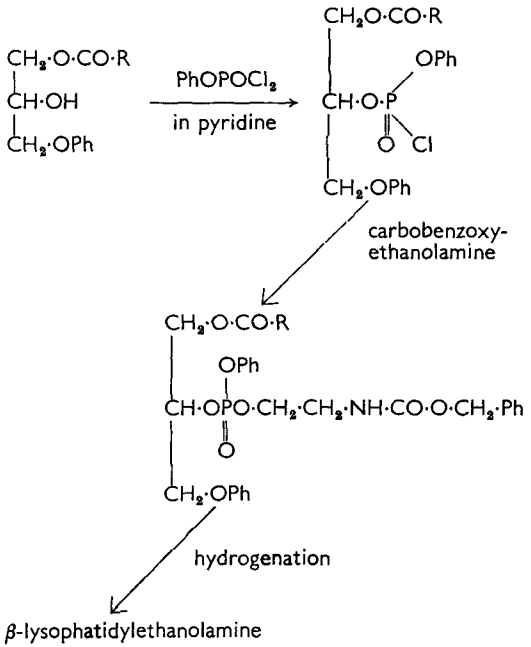
1. Cytidine triphosphate + phosphorylethanolamine ⇌ Cytidine diphosphate ethanolamine

2. ⫽ diacyl glycerol → phosphatidylethanolamine

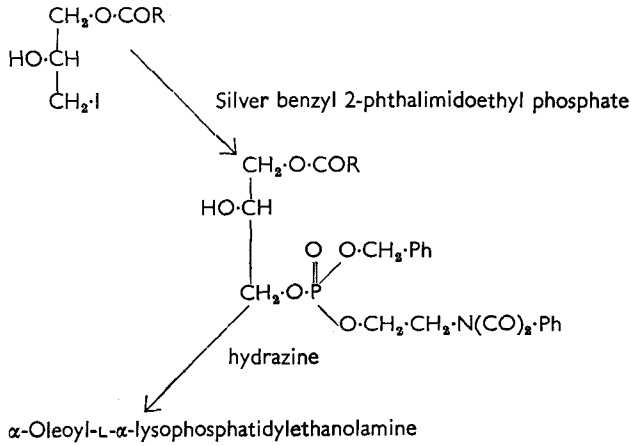
Later work (Kennedy, Borkenhagen and Smith, 1959) with de-oxy cytidine precursors gave slower reactions and poorer yields.

Lysophosphatidylethanolamine

Bayliss and others (1955) first synthesized a lysophosphatidylethanolamine by commencing with a monoglyceride, and following the method of Baer and others (1951) for phosphatidylethanolamine.



Following the conclusion that naturally occurring lysophatidylethanolamine has the fatty acid in the α-position, de Haas, van Deenen and Daemen (1962) prepared α-lysophatidylethanolamine commencing with γ-oleoylglycerol-L-α-iodohydrin:



However, the product contained a mixture of other compounds. Pure lysophosphatidylethanolamine was obtained by chromatographic separation of the final mixture.

PHYSICAL PROPERTIES

General

Phosphatidylethanolamine is a white hygroscopic powder that rapidly darkens on exposure to air to give a dark brown sticky mass, due to autoxidation (Lea, 1955). It is soluble in methanol, chloroform, ethanol, glacial acetic acid, ether and benzene, but insoluble in acetone. It is optically active, the specific rotation in chloroform at 20° being $+6{\cdot}1°$ (de Haas and van Deenen, 1961b). Lysophosphatidylethanolamine is a gleaming white, non-hygroscopic powder, and does not change colour on storage (Levene, Rolf and Simms, 1924). It is less soluble in organic solvents than phosphatidylethanolamine (Robins and Thomas, 1963b). The specific rotation of α-oleoyl glycerol-β-phosphorylethanolamine in chloroform at 20° was $-2{\cdot}5°$ (de Haas, van Deenen and Daemen, 1962).

Aqueous Solutions

Both substances form stable hydrophilic sols with water, whose turbidity increases with concentration. The sols are precipitated by small concentrations of potassium and calcium chlorides (Robins and Thomas, 1963a,b). Spiegel-Adolf (1935) indicated that phosphatidylethanolamine acted as a protective colloid for cholesterol against precipitation by salts and proteins, and protected colloidal gold from flocculation by neutral salts.

Phosphatidylethanolamine exists in water as a colloidal electrolyte and forms micelles in which the molecules are arranged in double layers with the polar groups orientated towards the aqueous medium and the hydrocarbon chains away from the micelle surface. Such bimolecular leaflets have a thickness of 30–60Å depending on hydrocarbon chain length, temperature and hydration (Engstrom and Finean, 1958). Electron microscopic examination of phosphatidylethanolamine in 0·01M ammonium carbonate solution, showed particles that were round or irregular flat discs having a width of 50–70Å (Wallach, Maurice, Steele and Surgenor, 1959).

By means of X-ray diffraction studies, Schmidt and Palmer (1940) found that the spacings between the leaflets in phosphatidylethanolamine sols were considerably larger than those in the dry substance, indicating the presence of water molecules between the leaflets.

Low angle diffraction studies by Finean and Millington (1955) showed that the spacing of phosphatidylethanolamine in the bimolecular leaflet decreased, usually in well defined steps, with rise in temperature. They concluded that this was due to tilting of the long chain molecules, as a result of the change of intermolecular Van der Waals and ionic forces.

Robins and Thomas (1963a, b) showed that both substances have marked surface-active properties, 0·01 per cent w/v aqueous sols reducing

the surface tension of water to 30 dynes/cm. The critical micelle concentration of phosphatidylethanolamine was in the range 0·002 to 0·01 per cent w/v, while that of lysophosphatidylethanolamine was in the range 0·001 to 0·002 per cent w/v. Surface tension studies indicated that both substances showed a large surface-ageing effect. These large changes of surface tension of lysophosphatidylethanolamine with time have been related to an intramolecular migration of the phosphate-ethanolamine moiety from the γ- to the β-position.

Surface Films

Phosphatidylethanolamine will form monomolecular films on water, and from force: area measurements the molecular area of distearoyl phosphatidylethanolamine was found to be 36Å^2 (van Deenen, Houtsmuller, de Haas and Mulder, 1962). Monomolecular films have been employed to study lipid-protein complexes with a view to investigating the nature of cell membranes. Apparently the protein molecules are spread out along the polar interface of the phosphatidylethanolamine monolayer, the basic groupings of the protein binding with the phosphoric acid groupings of phosphatidylethanolamine (Doty and Schulman, 1949; Payens, 1960). Many factors such as pH, salt concentration, dielectric constant, affect these associations, which alter the hydrogen bonding, electrostatic and Van der Waals forces between the protein and the lipid (Fraser, 1957).

Alexander, Teorell and Arborg (1939) studied the effect of various salts on monolayers of phosphatidylethanolamine. At the air: water interface there was little effect, but at the benzene: water interface calcium ions greatly stabilised the lipid in the interface. Rosano, Schiff and Schulman (1962) state that calcium ions link with phosphatidylethanolamine at the water: oil interface to form calcium cephalinate which prevents the transport of sodium and potassium ions across the interface. The calcium ion breaks up the phosphoric acid-amine polar group intramolecular association, and prevents intermolecular association between neighbouring molecules. However, no such interaction was detected with magnesium ions.

Acid-Base Relationships

Since the two substances contain a relatively weak basic group (ethanolamine) and a strong acidic group (phosphoric acid) they form zwitterionic structures in water. Various values for the iso-electric points have been estimated (Bull and Frampton, 1936; Garvin and Wallach, 1958; Wallach, Maurice, Steele and Surgenor, 1959; Payens, 1960) and have been found by surface tension measurements (Robins and Thomas, 1963a, b) to be at pH 3·1 for phosphatidylethanolamine, and at pH 3·25 for lysophosphatidylethanolamine.

Phosphatidylethanolamine has been shown to form zwitterions in water, whereas in solvents of low dielectric constant it exists as ions or neutral molecules (Fischgold and Chain, 1935). Jukes (1934) showed from electrometric titrations that phosphatidylethanolamine had two pK

values, at pH 1·1 and 8·9. Unlike lecithin, it bound alkali and exhibited a buffering capacity. The two pKa values for lysophosphatidylethanolamine have been quoted as 3·9 and 9·5 (Levene, Rolf and Simms, 1924). Phosphatidylethanolamine has been shown to bind inorganic cations over a range of pH, which suggested its possible role in biological electrolyte equilibria (Christensen and Hastings, 1940).

Blood Coagulation

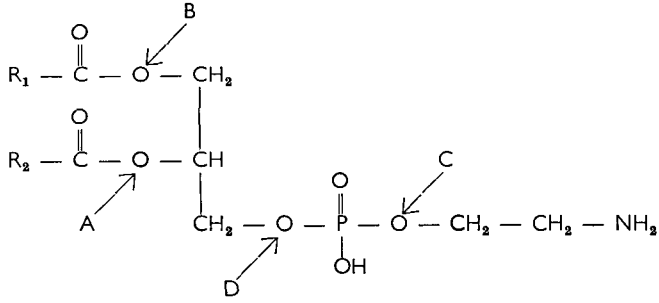
The cephalin group of phospholipids has been identified with the mechanism of blood coagulation. Thrombin formation was thought to be due to an enzymic mobilisation of cephalin and calcium at the colloidal surface of prothrombin (Wadsworth, Maltaner and Maltaner, 1931; Ferguson and Erikson, 1939).

Chargaff (1944) studied numerous phospholipid fractions from heart and brain tissue, and found that phosphatidylethanolamine showed only a slight thromboplastic activity. He concluded that thromboplastic active lipids could not be identified with any of the known phospholipids.

Poole and Robinson (1956) showed that phosphatidylethanolamine has a direct role in blood clotting. This activity was not due to lysophosphatidylethanolamine or contaminants since synthetic phosphatidylethanolamines had identical activity to phosphatidylethanolamine prepared from natural sources. This was later confirmed (Poole, Robinson and Harris, 1957). O'Brien (1956) indicated that phosphatidylethanolamine was contained in the platelets and is liberated when blood is shed.

Enzymatic Hydrolysis

There are four enzymes that can hydrolyse the phospholipids, and their points of attack are shown below.



The source and site of action of phospholipase A has already been discussed.

Phospholipase B removes the α-acyl grouping (Hanahan, 1955) but only from lysophosphatidylethanolamine: it has no action on phosphatidylethanolamine (Fairbairn, 1948).

Phospholipase C yields ethanolamine on hydrolysis of egg phosphatidylethanolamine and synthetic dimyristoyl DL-α-phosphatidylethanolamine, whereas synthetic β-phosphatidylethanolamine is only slightly attacked (Davidson and others, 1955).

A fourth enzyme, phospholipase D, hydrolyses phosphatidylethanolamine at the glycerol-phosphoric acid ester linkage, yielding phosphorylethanolamine. Until recently, the enzyme was considered to have no reaction upon cephalins (MacFarlane, 1948), but it has now been shown (de Gier, de Haas and van Deenen, 1961) that phosphatidylethanolamine emulsified with lecithins is susceptible to attack by the enzyme obtained from *Clostridium welchii*. These conditions of emulsification probably favour the requirements in charge and orientation of the molecules necessary for enzyme-substrate interaction. However, the phospholipase obtained from *Bacillus cereus* appeared to be active upon isolated and synthetic phosphatidylethanolamines. Since the mode of action of both bacterial enzymes were identical, the enzymes must differ in their distribution of charge.

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