THE STRUCTURE OF THE PHOSPHOLIPIDS[1, 2]

EARL B. WORKING

Kansas Agricultural Experiment Station, Manhattan, Kansas

AND

A. C. ANDREWS

Department of Chemistry, Kansas State College, Manhattan, Kansas

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I. INTRODUCTION

Fourcroy (13) in 1793 was probably the first to find indication of the occurrence of complex fatty compounds. About nineteen years later Vauquelin (58) isolated from the brain fatty materials that contained phosphorus. In the next few years this type of compound was obtained by other investigators from various sources, nearly all of which were animal in nature. These preparations were in all probability very impure mixtures of substances now known as galactolipids, lecithins, cephalins, and sphingomyelins.

Strecker (49) identified the nitrogen base in these fatty compounds as the same type of nitrogen compound he had previously discovered in bile and gave it the name of choline. The rest of the structure of the molecule was furnished by Diaconow (8) and Strecker (50), when they showed that two molecules of fatty acid, either alike or different, and a molecule of glycerol could be isolated from the hydrolytic products of these fatty

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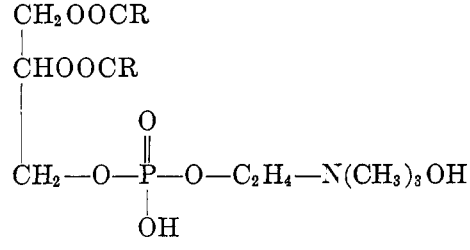
compounds. The fatty acids were thought to be esterified with the glycerol.

Of all the different phospholipid materials that have been found in animal tissue, three compounds of particular interest and importance have been isolated and studied,—namely, lecithin, cephalin, and sphingomyelin. It is with these three that this paper will deal. The structure and properties of these compounds were thoroughly covered in a classical monograph by MacLean and MacLean (39), and since that time not a great deal of change is to be found in the understanding of their structure, with the possible exception of cephalin.

II. LECITHIN

Diaconow (8) and Strecker (50) satisfactorily showed that the lecithin molecule consisted of two fatty acid radicals esterified to glycerol and a phosphoric acid radical attached both to the third hydroxyl group of the glycerol and to the nitrogenous basic group choline. These investigators did not agree, however, as to the manner in which the choline was attached to the phosphoric acid radical. The combination could be accomplished in two ways,—either through the hydroxyl group of the choline nitrogen as a salt, or through the hydroxyl of the ethanol group of the choline as an ester. The ester form of choline union, championed by Strecker, was later confirmed by Hundeshagen (21) and Gilson (15). The structure commonly accepted agrees in general with that given by MacLean and MacLean (39) as:



where the R groups are fatty acid radicals.

In this formula the β-carbon atom of the glycerol is asymmetric, and thus the compound should be optically active. Ulpiani (57) showed that lecithin is optically active. Three years later Willstätter and Lüdecke (59) isolated from the hydrolytic products of lecithin an optically active glycerophosphosphoric acid. This optical activity would seem to indicate that the above structure for lecithin is correct, rather than that of the possible isomer in which the cholinephosphoric acid radical is attached to the β-carbon atom of glycerol. This latter structure, however, may also occur in nature. Fischgold and Chain (10) indicate the possibility

that lecithin exists as the endo salt, in which the hydroxyl of the choline nitrogen and that of the phosphoric acid group lose a molecule of water. They find that in benzene–alcohol solution lecithin combines with one equivalent of hydrogen ions in acid solution.

The products of the hydrolysis of lecithin have been shown to be choline, phosphoric acid, glycerol, and fatty acids. The complete separation of lecithin from cephalin has been difficult to carry out, but when it is successful, the lecithin contains no trace of amino nitrogen, leaving choline as the sole base present. The presence of glycerol in lecithin was proven much earlier, since the complete separation from cephalin was not necessary. Gobley (16) in 1850 proved the presence of glycerol in the lecithin from certain fish eggs, and later Foster (12) found that the amount of glycerol present conformed to the theory. As to phosphoric acid and its proportion, the results of the older investigators are acceptable. Here again, the presence of cephalin in the samples of lecithin does not appreciably change the phosphorus content.

Much has been written about the fatty acid content of lecithin. Samples of lecithin from various sources, as brain, liver, egg yolk, and soybean, have been shown to contain identical saturated fatty acids,—palmitic and stearic. Among the unsaturated fatty acids found were oleic, linolic, arachidonic, and linolenic. The amount of saturated fatty acid was usually about equal to the amount of unsaturated fatty acid found; however, Sinclair (48) has shown that the proportion of unsaturated fatty acid in animal lecithins can be greatly increased by feeding a diet high in unsaturated fatty acids.

A zwitter-ion structure has been proposed for lecithin as well as for the other phospholipids. Thus it would be expected to show varying electrophoretic velocities in solutions of varying pH. Recently, Bull and Frampton (2) determined the isoelectric points of lecithin containing various amounts of cephalin, and found the pH to be dependent upon the amount of cephalin present as determined by the amino nitrogen content. Their isoelectric point for pure lecithin upon extrapolation of experimental values was at pH 6.4. This agrees well with that found by Chain and Kemp (3), who report a pH of 6.7 $\pm$ 0.2. A calculation of the isoelectric point from dielectric constant data places it at pH 7.5. The discrepancy here is attributed by them to decomposition of the lecithin, but Bull and Frampton question this conclusion. This spontaneous decomposition of lecithin, even when air was excluded, was reported by Price (44). Fischgold and Chain (10) found that the electrophoretic mobility increased with increased age of the lecithin suspensions. They also investigated the acid- and base-binding capacities of lecithin and other phospholipids in benzene–alcohol solution, finding that in alkaline solution

only those containing an amino group (the cephalin) can give off one equivalent of hydrogen ions. In acid solution, all the phospholipids were found to take up one equivalent of hydrogen ions. From this they assume that lecithin can exist only as a zwitter ion or as a cation. They strongly discount the existence of the "hydrate formula." Thudichum (56), Koch and Pike (28), Koch and Todd (29), and Peters and Man (43) have reported combination of ions with phospholipids. Christensen and Hastings (6) report on extensive studies of the binding capacities of phospholipids and inorganic salts. They find that fresh lecithin in aqueous solution does not bind sodium, potassium, or chloride ions. Lecithin showed no buffering over a pH range of 3 to 9 by electrometric titration.

In the several attempts to synthesize lecithin, Hundeshagen (21) obtained choline distearylglycerophosphate by treating distearylphosphoric acid with choline carbonate. This product was a choline salt and not the ester. Later Grün and Kade (18) obtained the choline chloride ester of distearylphosphoric acid (lecithin chloride), which agreed with lecithin in general solubility in many organic solvents but was obtained in insufficient quantity for positive identification. Grün and Limpächer (19) synthesized a distearyllecithin and established its constitution. No natural lecithin of this composition had been isolated; however, it had been prepared by hydrogenating purified lecithin from egg yolk. Recently Kabashima (22) obtained a dipalmitoyl-β-lecithin which showed many of the solubility properties of natural lecithin. Kabashima (23) also prepared a lysolecithin that resembled the natural product but possessed a somewhat smaller hemolytic action.

III. CEPHALIN

Apparently the first clear differentiation of cephalin from lecithin was made by Thudichum (56), who separated them by the insolubility of cephalin in alcohol. He considered the essential constituents to be cephalic acid, stearic acid, glycerophosphoric acid, and choline (see MacLean's (39) conclusion that Thudichum's "neurin" was properly choline) and suggested two possible structures. One of these, supported by the tendency of part of the phosphorus to cling to the fatty acids after hydrolysis, connected the fatty acids and glycerol directly to the phosphoric acid, with the choline replacing a hydroxyl group in the glycerol. The other structure was like that now accepted for both lecithin and cephalin. Cephalic acid, a fatty acid with seventeen carbon and three oxygen atoms, was proposed as an indispensable constituent, with various cephalins possible as a result of the replacement of the stearic acid by lower saturated acids or by unsaturated acids. Aminoethanol and another base not clearly defined were found, but were considered as probable decomposition products of the choline.

Stearic acid has been amply confirmed in cephalin, and is believed by Cousin (5), Parnas (41, 42), Levene and West (34), MacArthur and Burton (37), Levene and Rolf (33), and Klenk (25) to be the only saturated acid present in substantial quantities in brain cephalin, although traces of palmitic acid were found. Fränkel and Dimitz (14) reported considerable quantities of palmitic acid, but the soundness of their conclusion is questioned by Parnas. Nishimoto is quoted by Klenk and Schuwirth (27) as reporting considerable quantities of palmitic acid in egg cephalin.

Apparently the cephalic acid reported by Thudichum (56) was a mixture of partially oxidized unsaturated acids, and in view of the preponderance of unsaturated acids, it is not strange that he considered it the most important group. Parnas (41) probably did the first extensive work on the identification of the unsaturated acids. He concluded that linolic acid was the chief unsaturated acid present, and that the small quantities of oleic acid and of an acid with three double bonds found were probably derived from impurities. MacArthur and Burton (37), however, reported that oleic acid was present as about 50 per cent of the total fatty acids, and also observed indications of acids containing three and four double bonds. Support for their contention was afforded by Levene and Rolf (33), who isolated oleic and arachidonic acids and found evidence of some other unsaturated acids which they failed to identify positively. Klenk (25) hydrogenated the unsaturated acids of brain cephalin and found only stearic and behenic acids. He questioned whether Levene and Rolf might have been mistaken in claiming arachidonic acid. Klenk's experiments indicated that the twenty-two-carbon-atom unsaturated acid had at least four, possibly five, double bonds, and he suggests that it may be the clupanodonic acid of Tsujimoto. Nishimoto's results (see Klenk and Schuwirth (27)) on egg cephalin agreed with those of Levene and Rolf on brain cephalin in that he reported oleic and arachidonic acids. Sinclair's (48) work showed that one unsaturated acid may replace not only another unsaturated acid but also a saturated acid in cephalin as well as in lecithin, and it is quite generally accepted that different samples of cephalin differ in the fatty acids present. The wording of Sinclair's conclusions encourages a misinterpretation of his meaning. His first statement is clear: "One class is composed of the more highly unsaturated phospholipids, and functions in the essential make-up of the cell; the other class, consisting of the less unsaturated phospholipids, functions as an intermediary product in the metabolism of fat." The confusion is likely to enter when he describes the more highly unsaturated compounds as the "non-metabolic type." These phospholipids, chiefly cephalins, are undoubtedly highly important in cell metabolism; Sinclair is merely pointing out that the other type apparently functions primarily in fat metabolism.

More critical is the controversy over the nitrogen base in cephalin. Thudichum (56) thought it was choline, and Cousin (5) held that view as late as 1907, but practically all other workers in the field believed aminoethanol to be the base of cephalin, and that any choline found came from lecithin as a result of the intersolubility of the phospholipids. Such reviews as those of Mathews (40), MacLean and MacLean (39), and Thierfelder and Klenk (55) reflect this view, but mention the work of MacArthur (36) in which he reported the presence of an amino acid, supported by a copper method. Mathews (40, page 592) quoted MacArthur as finding aminoöxybutyric acid and serine, but did not give the basis for the conclusion or a specific reference. Syntheses by Grün and Limpächer (20) and by Kabashima (22, 23), using aminoethanol, gave products sufficiently similar to natural cephalin to be considered by them as identical, and Rudy and Page (46) obtained, by special preparation from natural cephalin, a product which doubtless had only aminoethanol as its base. With cephalin the divergence of the elementary analysis from that calculated for the accepted formula has been much greater than has been the case with lecithin. The explanations offered for this discrepancy have been reviewed by MacLean and MacLean (39) and by Thierfelder and Klenk (55). Rudy and Page (46), by a careful fractionation of the mother liquor of ordinary cephalin, obtained a product which agreed with the theoretical composition. This product should correspond, however, to the material containing ethanolamine which Darragh and MacArthur (7) and MacArthur, Norbury, and Karr (38) found in their lecithin fractions, and not to typical cephalin, which has always been identified by its insolubility in alcohol.

Gray (17) presents a well-conceived survey of the differences between experimental and theoretical values for the analysis of cephalin. He concludes that the discrepancy may be accounted for by an additional group or groups low in carbon and hydrogen and rich in oxygen. He gives considerable evidence for the presence of such groups, but fails to identify definitely any such compound. He notes particularly that reduced cephalin is non-hygroscopic, while the reduced synthetic compounds of Grün and Limpächer (20) and of Kabashima (22, 23) were hygroscopic.

Christensen and Hastings (6), from a study of the electrophoresis and the titration of cephalin, concluded that both the titration behavior and the instability of cephalin point towards a structure other than the generally accepted one. Their observation that about 0.6 equivalent of potassium or sodium is bound per mole of cephalin may be significant.

Folch and Schneider (11) investigated the base by means of the ninhydrin–carbon dioxide method and the periodate reaction, proving the presence of a hydroxyamino acid. The identification of glycolic acid in

the products from the reaction with ninhydrin and its isolation as the dimedon compound indicate that the amino acid present is serine. In confirmation, treatment with chloramine-T also yielded glycolic acid, which was isolated as the phenylosazone. Forty to 70 per cent of the nitrogen present in various samples was found to be amino acid nitrogen.

Folch and Schneider find 40 to 70 per cent of their cephalin preparation to consist of a cephalin with an amino acid base. Christensen and Hastings, using cephalin prepared in a very similar manner, find that about 0.6 equivalent of potassium or sodium is found per mole of cephalin. Hence it is entirely possible that it is the amino acid cephalin which binds the base and shows the buffering action believed by the latter authors to be characteristic of cephalin. If reference then be carried back to the discussion of Gray (17), and correction made for serine instead of aminoethanol, the calculated percentage of carbon comes somewhat closer to

TABLE 1

Analytical figures for cephalin (calculated and observed)

MATERIAL	C	H	N	P
	per cent	*per cent*	*per cent*	*per cent*
Calculated for stearyllinoleylcephalin, aminoethanol base	66.04	10.71	1.88	4.16
Calculated for stearyllinoleylcephalin, serine base	63.96	9.97	1.77	3.93
Calculated for potassium salt of foregoing	60.94	9.50	1.69	3.75
Observed values, average by Gray	60.41	9.79	1.67	3.59
Observed values, preparation by Folch and Schneider	60.0		1.605	3.89

that observed, but still the correspondence is not good. If, however, the serine cephalin be considered as combined with one equivalent of potassium, the agreement is almost perfect, as shown in table 1, copied after Gray with the addition of the data indicated. The analysis of the cephalin investigated by Folch and Schneider, which was purified in an exceptionally careful manner, is included. Gray found 12 per cent ash in his material, and since only a small part of this is explained as phosphorus, the assumption that a metal is present seems justified. Christensen and Hastings found 1.83 per cent potassium and 0.80 per cent sodium in the cephalin prepared according to the method of Levene and Rolf.

Folch and Schneider offer two explanations of their data: "There may be two cephalins preformed in the brain; or the ethanolamine-containing cephalin may be an artifact, originating post mortem by decarboxylation of the serine constituent." Figure 1 illustrates the decarboxylation suggested. Considering the wide divergence in properties of the two bases,

it seems unlikely that the name "cephalin" will be retained for these two compounds, and evidence seems to be gathering toward the support of the suggestion that the compound containing ethanolamine is a decomposition product.

Many workers have observed, and MacLean and MacLean (39) have discussed in particular, the fact that only when fresh tissue is obtained and dried quickly with avoidance of high temperatures is it possible to obtain a good yield of lecithin free from amino nitrogen. If the tissue is not fresh, or is not dried rapidly, a large amount of aminoethanol "cephalin" is found with the lecithin and is separated from it only with great difficulty. The obvious assumption would be that the lecithin is changed to cephalin, but this would require a demethylation of the lecithin, which scarcely seems possible.

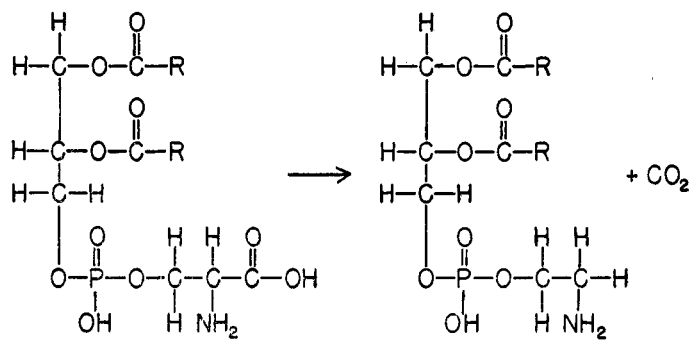


FIG. 1. Possible structure for cephalin

However, the anomaly clears up if we have lecithin and serine cephalin in the original tissue, since the serine cephalin precipitates readily and clearly with alcohol, and so is separated easily from lecithin. It is easy to understand that in old tissue, or during a slow drying, the serine could be decarboxylated. The aminoethanol "cephalin" thus formed is difficult to separate completely from lecithin, owing to its slight solubility in alcohol and considerable solubility in alcohol solutions of lecithin.

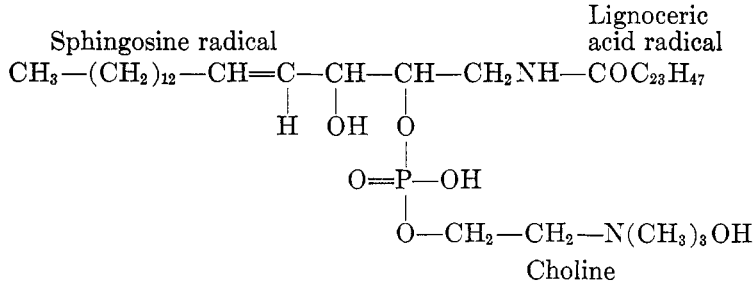
If cephalin does contain an amino acid, the carboxyl should account for the alkali-binding observed by Christensen and Hastings, and the zwitter-ion structure between the amino group and the phosphoric acid is still possible.

IV. SPHINGOMYELIN

Thudichum (56) was the first to isolate a white phospholipid material from a warm alcohol extract of brain tissue upon cooling. This white

material is sphingomyelin and differs from the other phospholipids in being easily crystallized. Thudichum recognized the structure to be a diaminomonophosphatide with a nitrogen to phosphorus ratio of 2 to 1. It contains no glycerol.

Upon hydrolysis, sphingomyelin was shown by Thudichum to yield the two bases, choline and sphingosine, and fatty acids. It was generally believed that the natural sphingomyelins consisted of the cholinephosphoric acid ester of sphingosine. The sphingosine amino group combined with fatty acids through a ceramide link (NH—CO), leaving one hydroxyl group of the sphingosine free. The fatty acid was thought to be lignoceric, stearic, or palmitic. Thudichum at one time believed the molecule to contain a higher alcohol ($C_{18}H_{35}O_2$), which he called sphingol. This has now been shown to be absent. Levene succeeded in isolating a hydrolytic product of sphingomyelin which upon complete reduction with hydrogen in presence of palladium had the composition of lignocerylsphingine. This compound formed no salts with ordinary mineral acids, and liberated no nitrogen when treated with nitrous acid, thus indicating the absence of free amino groups in the molecule. Levene considered the amino group as the linking group between the sphingine radical and the lignoceric acid radical. The structure suggested by Levene for sphingomyelin and still accepted as essentially correct is:



Recently, Thannhäuser and Reichel (52) reinvestigated the problem of the structure of sphingomyelin by varying the means of hydrolysis. By hydrolysis of the choline–phosphoric acid link by liver phosphatase, lignocerylsphingosine, phosphoric acid, and choline were isolated, and palmitic acid was the only fatty acid found. These investigators conclude that the fatty acid–amide link was not broken and that the palmitic acid could only have been linked to the sphingomyelin molecule as an ester with the second hydroxyl group by the sphingosine radical. Further, sphingomyelin was also hydrolyzed with lipase, and again the same fatty acid ester bond was broken. Mild hydrolysis with alkali gave palmitic acid and unesterified lignocerylsphingomyelin. So Thannhäuser and

Reichel conclude that natural sphingomyelin is probably a mixture of lignocerylsphingomyelin and lignocerylsphingomyelin fatty acid ester. Only in this way could they account for the presence of free fatty acids and lignocerylsphingomyelin among the split products of enzymatic hydrolysis. As a result of their analysis, they give $C_{47}H_{97}N_2PO_7$ as the empirical formula for lignocerylsphingomyelin. The observed percentage of nitrogen was 3.32 and that of phosphorus was 3.97, in comparison to calculated percentages of 3.36 and 3.73, respectively. In a continuation of their work, Thannhäuser and Reichel (55) synthesized various lignocerylsphingosine fatty acid esters, sphingosine fats, and sphingosine amides, using various fatty acids. The successful synthesis of these compounds shows that both hydroxyl groups of sphingosine can be esterified.

Until 1929, sphingosine was considered to have seventeen carbon atoms with a double bond between the fourth and fifth. This was believed to be the case after Lapworth (30), Levene and West (35), and Levene (31) found that tridecyclic acid (C_{13}) was formed upon oxidation of sphingosine at the double bond. However, Klenk (24), and Klenk and Diebold (26) in 1939 obtained myristic acid and α-aminobutyric acid upon oxidation at the double bond with ozone and subsequent hydrolysis. Thus a chain of eighteen carbon atoms with an α-amino group and a double bond between carbon atoms 4 and 5 was indicated.

Sphingomyelin then is a phospholipid without glycerol as a foundation. Rather, the two hydroxyl groups and the one amino group on the terminal carbon atoms of sphingosine act as the linking groups to the attached radicals. The ceramide link to the lignoceryl acid radical is on the α-carbon atom, the cholinephosphoric acid radical is probably on the β-carbon atom esterified through the hydroxyl group, and the second hydroxyl group on carbon atom 3 may or may not be esterified to a fatty acid,—palmitic or possibly stearic. Carbon atoms 3 and 4 are asymmetric, and sphingomyelin is accordingly optically active. Recently, Thannhäuser and Benotti (51) have shown that sphingomyelin has a monomeric form rather than the polymeric form previously suggested (52). Their conclusions were based on results of molecular weight determinations made through boiling-point measurements, titration with acid, and analysis of the silver and platinum salts.

Christensen and Hastings (6) recently examined sphingomyelin in regard to its property to bind various ions. This property has been suggested in the past as due to zwitter-ion formation in the sphingomyelin molecule when in solution. Chain and Kemp (3) determined the isoelectric point for sphingomyelin by the electrophoretic method as being at or near pH 6.0. Fischgold and Chain (10) found that in benzene–alcohol solution sphingomyelin bound one equivalent of hydrogen ions in acid reactions but did not yield hydrogen ions in basic solution except to a very small extent, which

was apparently due to impurities. This seemed to favor the zwitter-ion form for sphingomyelin when in solution. Christensen and Hastings (6) titrated sphingomyelin electrometrically, and found that it did not show any buffering action over wide ranges of pH; they failed to find any appreciable combination of sphingomyelin with chloride or sodium ions.

V. PLANT PHOSPHOLIPIDS

Thierfelder and Klenk (55) gave a very adequate review of the literature on plant phospholipids to up 1930, and no important additions to our knowledge of their structure have appeared in the literature since that time. The presence of sphingomyelin has not been proven in plants, and Austin (1) particularly mentions its absence in yeast. Plant lecithins and plant cephalins corresponding closely to animal lecithins and cephalins have been prepared, but they appear throughout to be much more difficult to purify, especially as to freedom from sugars. This is generally considered to be a contamination rather than a structural part of the lipid, since several workers have obtained materials apparently free from sugar (see Schulze (47), Rewald (45), and Diemair, Bleyer, and Schmidt (9)). The phospholipids of cereals appear to be especially low in nitrogen and phosphorus. Those from barley, wheat, and oats, prepared by Diemair, Bleyer, and Schmidt, are typical in that they contain only about 1.0 per cent of nitrogen and 2.2 per cent of phosphorus, although they are apparently free from sugar. Calcium salts of the phosphatidic acids, as described by Chibnall and Channon (4), could explain low values for nitrogen, but no satisfactory explanation has yet been offered for the low amount of phosphorus found in many plant phospholipids. It is not yet clear what other impurities might cling so firmly to them as not to be removed by the methods used successfully with materials of animal origin.

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