Cardiolipin and Other Phospholipids in Ox Liver

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The objects of this work were to find whether the polyglycerophosphatide detected in liver extracts had the same structure as cardiolipin isolated from ox-heart muscle, and to examine the fatty acid composition of the phospholipids. The molar ratios glycerol:phosphorus:fatty acid reported for the glycerophosphatide (McKibbin & Taylor, 1952; Dawson, 1958; Marinetti, Erbland, Albrecht & Stotz, 1957) differed from those found for cardiolipin by Pangborn (1947), who considered cardiolipin to be a fully acylated polyglycerophosphatide with four glycerol, three phosphoric acid and six fatty acid residues. However, evidence in favour of the structure bis(diacylglycerophosphoryl)-glycerol (diphosphatidylglycerol) for cardiolipin was obtained by oxidative degradation of the deacylated polyglycerophosphate, and by the identification of diglycerides and glycerol diphosphate as the main products of hydrolysis of cardiolipin in 90% acetic acid (Macfarlane, 1958; Macfarlane & Wheeldon, 1959).

The amount of polyglycerophosphatide isolated by McKibbin & Taylor (1952) was about 0.5% of the total phospholipid. Hanahan, Dittmer & Warashina (1957) fractionated the phospholipids of rat and ox liver on silicic acid and found about 2% of the total phosphorus in a fast-running nitrogen-low fraction and about 7% as inositide. While the present work was in progress, Faure, Morelec-Coulon, Maréchal & LeBorgne (1959) isolated from ox liver, by solvent fractionation, a polyglycerophosphatide with an iodine value and a serological activity similar to that of cardiolipin, and with molar ratios of glycerol:phosphorus:fatty acid of 3:2:4; they made no comment on the structure and considered that the acidic phospholipid fraction contained phosphatides other than cardiolipin and phosphatidylinositol. The amount of cardiolipin and inositide obtained by these authors was apparently equivalent to about 1.1 and 1.4% respectively of the total phospholipid phosphorus, on average figures for water and lipid-phosphate content of liver. At the same time Hübscher & Clark (1959) reported briefly the isolation from ox liver of phosphatidic acid, in a yield of 0.5–1.0% of the total lipid phosphorus; this is of considerable interest since, although other workers have not found phosphatidic acid in tissue

extracts, Hokin & Hokin (1958) detected its presence by heavy labelling with ^{32}P *in vivo*.

In an attempt to resolve some of the discrepancies, the acidic phospholipids were fractionated by chromatography on silicic acid and also by solvent fractionation of the free acids on the lines used by Faure *et al.* (1959).

By both methods, described below, cardiolipin was isolated in amounts equivalent to about 1–2% of the total phospholipid phosphorus, but unequivocal evidence for the presence of naturally occurring phosphatidic acid was not obtained. The discrepancy between these results and those of Hübscher & Clark (1959, 1960) is discussed later.

METHODS

Analytical methods for P, N, amino N, inositol, glycerol, fatty acid esters (colorimetric), fatty aldehyde, iodine values, cholesterol, phosphomonoester by enzymic assay and ester phosphatide were those used by Gray & Macfarlane (1958). The ratios of constituents are given on a molar basis.

Chromatography of phospholipids on silicic acid-impregnated paper was done according to Marinetti, Erbland & Kochen (1957) at 2° with diisobutyl ketone-acetic acid-water (40:20:3, by vol.). In this system the cardiolipin spot appears in front of and separated from the phosphatidylethanolamine spot, corresponding in position with spots 6 and 5, Fig. 1A of Marinetti, Erbland & Kochen (1957); the phosphatidylserine and phosphatidylinositol spots corresponded approximately with spots 4 and 1 in this Figure. Cardiolipin prepared from heart muscle and analysed samples of other phospholipids were used as markers. Paper chromatography of water-soluble phosphoric esters, obtained by mild alkaline hydrolysis, was carried out by Dr L. W. Wheeldon according to Dawson (1954). The relative R_F values in phenol saturated with 1% NH_3 in water were: glycerophosphorylinositol, 0.09; polyglycerophosphate from cardiolipin, 0.13; glycerophosphorylserine, 0.20; glycerophosphate, 0.25; glycerophosphorylethanolamine, 0.67; in *tert*-butanol-water (82:38, v/v), trichloroacetic acid (10%, w/v); glycerophosphorylinositol, 0.26; glycerophosphorylserine and glycerophosphorylethanolamine, 0.41; polyglycerophosphate from cardiolipin, 0.45; glycerophosphate, 0.61.

Chromatography of lipid extracts on silicic acid columns was done as in Gray & Macfarlane (1958). Gas chromatography of the methyl esters of fatty acid mixtures was carried out by Dr G. M. Gray; the saponification of the phospholipid and preparation of the methyl esters were done as described by Gray (1960).

Identification of the fast-running glycerophosphatide in silicic acid fractionation of ox-liver extract L₁ as cardiolipin

Minced ox liver (5.5 kg.) was extracted first with 2 l./kg. and then with 0.5 l./kg. of chloroform-methanol (1:1, v/v) by stirring for 10–15 min. at room temperature with cold solvent. The chloroform layer was washed three times with 0.5 vol. of water, cooled to -10° , filtered from ice and fluff and dried with CaSO_4 . The yield of lipid in the washed extract (L_1) was equivalent to 28.2 g. dry wt. and 890 mg. of P/kg. of fresh liver; P, 3.1 % dry wt.

Portions of L_1 containing 320 mg. of P were chromatographed on columns of 200 g. of Mallinckrodt silicic acid (100 mesh, diam. 5.5 cm., height 15 cm.). The columns were developed first with about 2 l. of chloroform, to remove the neutral lipid, and then with chloroform-methanol (95:5, v/v), to elute the low-N fraction (fraction A). The runs did not separate the N-free phospholipid cleanly; it was sandwiched between a slow tail of the non-P lipid and a fast-running front of the kephalin band, and some was undoubtedly lost in the kephalin front. The kephalin, main lecithin and mixed lecithin and sphingomyelin fractions were eluted by changing the solvent in stages up to chloroform-methanol (20:80, v/v); these fractions contained about 32, 1, 53 and 10 % of the total lipid respectively. [Phosphatidylinositol had been removed by the washing of the chloroform extract.]

Identification of cardiolipin in fraction A. Four lots of fraction A containing 18 mg. of P (a yield of 1.8 % of the total lipid P) were pooled. The material was very dark-coloured; P, 2.5 % on dry wt.; ratio Na:amino N:fatty acid ester:P, 0.11:0.12:1.7:1.0. After hydrolysis in 90 % acetic acid at 50° and addition of Feulgen reagent in the usual colorimetric estimation of fatty aldehyde, a purple colour developed which was not extracted into octan-2-ol but remained in particles at the interface. This atypical reaction is discussed below. A sample of fraction A was saponified in methanolic 0.2N-NaOH for 15 min. at 38° . Paper chromatography of the water-soluble ester showed a large spot corresponding to that of the polyglycerophosphate from authentic cardiolipin, small glycerophosphorylserine and glycerophosphoryl-ethanolamine spots and a faint glycerophosphate spot; treatment of the water-soluble ester with bone phosphomonoesterase liberated less than 5 % of the total P as orthophosphate; the content of phosphatidic acid, which gives rise to glycerophosphate on deacylation, was therefore low.

Fraction A (15 mg. of P) in 5.0 ml. of ether was brought to about pH 6 with ethanolic 0.2N-NaOH;

the solvent was removed *in vacuo* at low temperature and the residue redissolved in 3.0 ml. of ether and twice precipitated with acetone at -10° . The precipitate (P, 10 mg., ratio, amino N:P, 0.05:1.0) was dissolved in 5.0 ml. of ether and 50 ml. of ethanol was added with warming at 38° ; after 48 hr. at 2° the colourless solution AS (4 mg. of P) was recovered by centrifuging.

This ethanol-soluble sodium salt contained 3.7 % of P on dry wt.; ratio glycerol:P:fatty acid ester, 1.4:1.0:2.1. A portion (62 mg.) of AS was saponified in ethanolic 0.2N-KOH for 2 hr. at room temp. and at 2° overnight. The fatty acids recovered weighed 45 mg. (74 % on dry wt.); iodine value 132. The ethanol-insoluble potassium salt of the phosphoric ester was collected by centrifuging, washed once with ethanol, dried and dissolved in water; hydrolysis of a sample with phosphomonoesterase liberated less than 5 % of the P as orthophosphate; oxidation with NaIO_4 at 20° for 30 min. gave 0.97 mole of formaldehyde/mole of P. This agrees with the structure of three glycerol residues linked by two phosphoric acid groups (Macfarlane, 1958). On treatment with periodate at 100° in $\text{N-H}_2\text{SO}_4$ for 1 hr. the apparent content of glycerophosphate (Burmester, 1946) was 42 % of the total P; with prior hydrolysis of the ester in N-HCl at 100° for 10 min. the value was 91 %; this behaviour corresponds with that of the polyglycerophosphate from cardiolipin.

It was concluded that a polyglycerophosphatide, with a structure similar to that of cardiolipin, was the main phospholipid component in the fast-running fraction. This is in contrast to the findings of Hübscher & Clark (1960). The recovery of the soluble sodium salt, however, was low, and the ethanol-insoluble sodium salts had a lower ratio of glycerol:P, 1.2:1.0, and gave an atypical Feulgen reaction. The nature of this fraction is discussed later.

Recovery of acidic phospholipids as barium salts from methanol extracts

Ox liver was extracted twice by stirring for 10–15 min. with 1.2 l. of cold acetone/kg. at room temp. after mechanical blending with some of the solvent. The residue was extracted with 2 l. of methanol/kg. at 2° for 24 hr. with occasional shaking. Aqueous 1.5M- BaCl_2 was added to the methanol filtrate until no further precipitate formed, and the insoluble barium salts were collected by decantation and centrifuging, and washed with methanol. A measured part of the methanol supernatant, containing the bulk of the lecithin and kephalin, was evaporated at low temperature, and the residue was taken in chloroform-methanol (2:1, v/v) and shaken with water acidified with HCl; the chloroform layer was then washed

several times with water and dried with CaSO_4 . This fraction (C) was used for preparation of kephalin and lecithin. The crude barium salts were suspended in ether, and the ether-soluble acidic phospholipids (fraction A_1) were recovered by shaking at 2° with a mixture of conc. HCl -methanol-water, 1:4:50, and washing the ethereal solution with water. (Conversion of salts into the free acids in this way was always carried out at 2° , and as rapidly as possible, and the acids were reconverted into the barium or sodium salt for storage overnight at stages.)

The yield of lipid P was about 640–740 mg. of P/kg. of fresh liver, in three different preparations, (L_2 , L_3 , L_4) of which 17–22% was recovered in fraction A_1 . This fraction had a ratio amino N:inositol:P of about 0.5:0.33:1.0, and contained a varied small amount of choline due to lecithin carried down in the barium salts. The content of cardiolipin and other phosphatidic acids was therefore about 15% of A_1 , or about 3% of the total lipid P, and the content of phosphatidylinositol about 6–7% of the total.

Fractionation of acidic phospholipids from preparation L_2 on silicic acid

The free acid L_2A_1 in ether (2 mg. of P/ml.) was added to 9 vol. of methanol containing 5.0 ml. of 1.5M- BaCl_2 /l. The insoluble barium salts were washed once with methanol; the ratios amino N:inositol:P were 0.15:0.50:1.0. A portion was converted into the free acid A_2 (46 mg. of P) in ether; this was dried, dissolved in chloroform and immediately chromatographed on 60 g. of silicic acid. After passage of 100 ml. of chloroform and 200 ml. of chloroform-methanol (19:1, v/v) a sharp band I (7 mg. of P) was eluted; this fraction contained no inositol and had a ratio amino N:P of 0.03:1.0; a deacylated sample gave a glycerophosphate and a polyglycerophosphate spot on paper. This band tailed into an intermediate fraction II (5.0 mg. of P) with a ratio inositol:P of 0.5:1.0, and on changing the solvent to chloroform-methanol (9:1, v/v) another sharp band III (26 mg. of P) was eluted; this fraction had ratios amino N:inositol:P, 0.13:0.8:1.0, and a deacylated sample gave glycerophosphorylinositol and glycerophosphorylserine spots on paper. The remaining P (7 mg., ratio amino N:P, 0.60:1.0) was eluted with chloroform-methanol (4:1, v/v).

The front fraction A_2I was evaporated and dissolved in 3.0 ml. of ether; the solution was diluted with 33 ml. of ethanol (to give approximately a concentration of 0.5% of phospholipid) and brought to approx. pH 6.6 with ethanolic 0.2N- NaOH . A flocculent precipitate formed; the mixture was kept at 2° . A similar fraction (6 mg. of P) obtained by chromatographing another portion of A_2 was

added to it, and the ethanol-insoluble sodium salts were separated by centrifuging.

The ethanol-insoluble salts (? of phosphatidic acid) were taken up in chloroform and separated from a small residue containing 2.0 mg. of P, of which 1.2 mg. was water-soluble. The solution contained 3.0 mg. of P, with a ratio fatty acid ester:P of 1.0:1.0, and gave an atypical reaction with the Feulgen reagent; a deacylated sample gave 65–70% of the P as phosphomonoester by enzymic assay.

The ethanol-soluble sodium salts (cardiolipin fraction) contained 7.8 mg. of P (i.e. 60% of the fast-running fraction A_2I) with a fatty acid ester:P ratio of 3.4:1.0, indicating the presence of P-free lipid. The solution was evaporated at low temperature and the residue in 2.0 ml. of ether was treated with 6 vol. of acetone at -10° . The acetone supernatant contained 213 mg. of lipid, P, 1.1%. The precipitate weighed 107 mg.; P, 4.2%; fatty acid ester:P, 1.9:1.0; the Feulgen reaction (10 μg . of P) was very faint. A deacylated sample contained less than 5% of phosphomonoester and gave a large spot corresponding to that from cardiolipin.

The low fatty acid ester:P ratio in the ethanol-insoluble salts and the presence of P-free lipid in the ethanol-soluble fraction suggested that some breakdown had occurred.

Phosphatidylinositol. Fraction A_2III (25 mg. of P), obtained by silicic acid chromatography of A_2 , was dissolved in 15 ml. of ether and brought to approx. pH 6.6 by addition of ethanolic 0.2N- NaOH ; methanol (6 ml.) was added slowly until the solution remained turbid. A semi-crystalline deposit soon formed at room temperature and was collected after several days at 2° ; dry wt., 0.51 g.; P, 3.5%; ratios amino N:inositol:P, 0.06:0.90:1.0. The fatty acid composition of this sample of phosphatidylinositol (L_2A_2III) is shown in Table 3. The recovery of inositol in this fraction was equivalent to about 70% of the inositol in L_2A_2 .

Separation of phosphatidylinositol and cardiolipin by fractionation of the free acids with ethanol

The crude barium salts from a methanol extract (L_4) of liver were converted into the free acid L_4A_1 . A sample (4 μg . of P) chromatographed on silicic acid paper, and stained with Rhodamine G and ninhydrin, showed four spots, corresponding to cardiolipin, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. The free acid (840 mg. of P) in 300 ml. of ether was poured with stirring into 2 vol. of methanol containing 15 ml. of 1.5M- BaCl_2 : the insoluble barium salts were twice taken up in 250 ml. of ether and reprecipitated with 2 vol. of methanol, and then washed with acetone (A_3). The combined supernatants (B) contained mainly kephalin with a little inositide; the serine-ethanolamine ratio was 2.2:1.0.

The further fractionation of A₃ was based on the separation of the ethanol-insoluble acids (phosphatidylinositol) from the ethanol-soluble acids (cardiolipin and phosphatidic acid) and the separation of cardiolipin and phosphatidic acid by fractionation of their sodium salts in ethanol.

The composition of fractions derived from L₄A₁ in the following procedure is shown in Table 1.

The purified barium salts A₃ were converted into the free acid A₄ and treated in dry ether (15 ml.) with 100 ml. of ethanol; the flocculent precipitate was redissolved in 12 ml. of ether and treated with 60 ml. of ethanol. The ethanol-insoluble acids (A₅) were dissolved in ether and the barium salts precipitated by addition of BaCl₂ and methanol; phosphatidylinositol was recovered from this fraction (see below). The combined ethanol-soluble acids A₄S were diluted to 300 ml. with ethanol and brought to approx. pH 6.6 with ethanolic 0.2N-NaOH. It was calculated that this volume was sufficient to keep the sodium salt of cardiolipin in solution.

This fractionation gave a substantial recovery of phosphatidylinositol in A₅, and the ethanol-soluble acids were enriched in cardiolipin or other phosphatidic acids, but the carry-over between the fractions was considerable (Table 1).

Fractionation of ethanol-soluble acids as sodium salts

Ethanol-soluble sodium salts. On neutralization of the solution A₄S a precipitate (A₆) formed which was removed after 48 hr. at 2° from the ethanol-soluble sodium salts A₇ (40 mg. of P). A deacylated sample of A₇ gave spots corresponding to polyglycerophosphate and glycerophosphorylserine by paper chromatography, and contained about 3% of phosphomonoester by enzymic assay. The fatty acid ester:P ratio, however, was 2.7:1.0, indicating

the presence of P-free lipid. The material was chromatographed at room temperature on silicic acid (50 g.) as the sodium salt in chloroform. Passage of 400 ml. of chloroform removed some P-free lipid (380 mg. containing approx. 1000 μ-moles of fatty acid ester). The phospholipid was then eluted with chloroform-methanol (1:1, v/v) and dissolved in ethanol. The solution was acid and was adjusted to approx. pH 6.6 with ethanolic NaOH, and after 48 hr. at 2° the solution A₇S (35 mg. of P) was decanted from a small deposit. The solution had fatty acid ester:amino N:P ratios, 1.9:0.08:1.0; inositol, not detectable; P, 3.9% on dry wt. On silicic acid paper, spotting 4 μg. of P, it gave a large cardiolipin spot and a small phosphatidylserine spot. The fatty acid composition of this sample of cardiolipin (L₄A₇S) is shown in Table 3.

Ethanol-insoluble sodium salts of ethanol-soluble acids. These salts (A₆) by analysis contained 40% of phosphatides other than phosphatidylinositol and kephalin. A deacylated sample on paper chromatography gave spots corresponding to polyglycerophosphate, glycerophosphorylinositol and glycerophosphorylserine and also a weak glycerophosphate spot. It may be noted that this glycerophosphate spot was produced from material which had not been chromatographed on silicic acid. The material (80 mg. of P) was chromatographed as the sodium salt in chloroform on 65 g. of silicic acid, and the fast-running fraction (35 mg. of P), eluted with chloroform-methanol (94:6, v/v), was dissolved in ether (12 ml.) and ethanol (180 ml.) and adjusted to pH 6.6 with ethanolic NaOH. A precipitate (A₈P) formed immediately and was removed after 48 hr. at 2°. The supernatant material A₈S (20 mg. of P) now recovered as sodium salts soluble in ethanol contained no inositol, and had ratios fatty acid ester:amino N:P of 2.2:0.03:1.0;

Table 1. *Ethanol fractionation of acidic phospholipids*

The fractions were derived from the crude barium salts from the methanol extract L₄ of ox liver, as described in the text. Fraction A₁ contained 22% of the total phospholipid P in L₄.

Fraction	Nature	P in fraction		Distribution of P (% of total in fraction)		
		(mg.)	(% of total)	Inositide	Kephalin	Other compounds
A ₁	Ether-soluble acid from crude barium salts	840	100	33	50	17
A ₄	Ether-soluble acid from purified barium salts A ₃	520	62	42	32	26
B	Supernatants from A ₄	285	34	6	86	8
A ₄ S	Ethanol-soluble acid from A ₄	135	16	30	14	56
A ₆	Ethanol-insoluble sodium salts from A ₄ S	88	10.5	43	17	40
A ₇	Ethanol-soluble sodium salts from A ₄ S	40	4.8	0	11	89
A ₈ S	Ethanol-soluble sodium salts from silicic acid fraction of A ₆	20	2.4	0	5	95
A ₈ P	Ethanol-insoluble sodium salts from silicic acid fraction of A ₆	14	1.7	12	11	77
A ₅	Ethanol-insoluble acid from A ₄	380	45	48	35	17
A ₉	Crystalline inositide from A ₅	168	20	90	11	0

a deacylated sample contained less than 3% of phosphomonoester. Chromatography of A_3S on silicic acid paper (4 μ g. of P) showed a large cardiolipin spot, faint phosphatidylethanolamine and phosphatidylserine spots, and a faint lipid spot in front of the cardiolipin spot. The fatty acid composition of this cardiolipin (L_4A_3S) is shown in Table 3. The recovery of cardiolipin in A_7 and A_8S was 60 mg. of P. It may be noted that in the presence of impurities the solubility of cardiolipin was low.

'Phosphatidic acid' fraction. The fraction A_6P , the ethanol-insoluble sodium salts of ethanol-soluble acids, had a fatty acid ester:P ratio of only 1:1, corresponding to a lysophosphatide; ratios amino N:inositol:P, 0.11:0.12:1.0; P, 4.9% on dry wt. A deacylated sample contained 75% of phosphomonoester by enzymic assay. On silicic acid paper, staining with Rhodamine G, A_6P (4 μ g. of P) gave weak spots for phosphatidylserine and phosphatidylethanolamine, no cardiolipin spot and a faint spot, similar to that in A_3S , in front of the cardiolipin spot, but these spots obviously only accounted for a small part of the material spotted, and much apparently remained at the origin.

On treatment of a sample of A_6P (10 μ g. of P) with 90% acetic acid at 50° and addition of Feulgen reagent a deep-purple colour developed which, unlike that produced by fatty aldehydes, was not extractable into octan-2-ol; an old sample of triolein behaved similarly. A portion of A_6P (216 μ moles of P) was saponified in methanolic 0.2N-KOH 15 min. at 38°; after acidification the fatty material was extracted into ether (wt. 82 mg., equivalent to 290 μ moles of mol.wt. 284) and then refluxed in methanolic 0.8N-HCl to convert fatty acids and any aldehydes into methyl esters and acetals. The product was extracted into ether and washed as usual with $NaHCO_3$ solution and water; wt. 78 mg. This product, which gave a strong Feulgen reaction, was examined by gas chromatography; judging from the area of the peaks, only about 67% was present as volatile substances. These were accounted for as the methyl esters of stearic acid (35%), linoleic acid (44%), oleic acid (10%), linolenic acid (3%), eicosatrienoic acid (1.5%) and small amounts (less than 1%) of C_{13} , C_{14} , C_{15} , C_{16} and C_{17} acids.

It is concluded that the Feulgen reaction given by this fraction is not due to any plasmalogen derivatives, but is analogous to the Kreis reaction with phloroglucinol, and that the fraction, which approximates in composition to a lysophosphatidic acid but contained both nitrogen and inositol, is, in part, a conglomeration of degraded or complexed and 'rancid' phospholipids; these could be derived either from plasmalogens or ester phosphatides, owing partly to the lability towards acid

and partly to the auto-oxidation of the highly unsaturated acids present in the inositide and ester phosphatides. The presence of naturally occurring phosphatidic acid in A_6P is of course not excluded, but since, in view of the anomalous reactions and the suspicion of breakdown, it appeared impossible to conclude that any phosphatidic acid positively identified was in fact natural, no attempt was made at further fractionation. The amount of P recovered in A_6P was 1.7% of the P in the crude acidic phospholipid A_1 and 0.4% of the total lipid P.

Phosphatidylinositol. The ethanol-insoluble acids A_5 (320 mg. of P) were recovered as barium salts insoluble in methanol and reprecipitated from ether solution with methanol. The methanolic supernatant contained 102 mg. of P; ratio amino N:P, 0.8:1.0. The barium salts were converted into the sodium salts (180 mg. of P) insoluble in ethanol; these were dissolved in ether (100 ml.) and the solution was treated with methanol until turbid; the semi-crystalline deposit, A_5 , contained 168 mg. of P; ratio amino N:inositol:P, 0.1:0.9:1.0; chromatography on silicic acid paper gave a large phosphatidylinositol and weak phosphatidylethanolamine and phosphatidylserine spots. The fatty acid composition (L_4A_5) is shown in Table 3. The recovery of phosphatidylinositol in L_4A_5 , based on inositol assays, was equivalent to 64% of the inositide in L_4A_1 .

Separation of kephalin and lecithin from the methanol-soluble fraction C of the extract L_3

A portion (79 mg. of P) of fraction L_3C , recovered after removal of the methanol-insoluble barium salts, containing the ester phosphatides and about 3% of sphingomyelin by assay of alkali-stable P, was chromatographed on 75 g. of silicic acid; after passage of 200 ml. of chloroform, which eluted a little P-free lipid, the solvent was changed successively to chloroform-methanol (9:1, 4:1 and 1:1, v/v), with which 90% of the ester phosphatides was eluted; the tail of lecithin and sphingomyelin was discarded. The eluate fractions were pooled, on the basis of P, amino N and fatty acid ratios, as shown in Table 2, the lecithin eluates being pooled in four fractions with decreasing iodine values. The kephalin and the first three lecithin fractions, which contained small amounts of plasmalogens, were treated individually with 90% acetic acid at 38°, and the free aldehyde, lysophosphatide and diacyl phosphatide were separated chromatographically by Dr G. M. Gray; the nature of the aldehydes and the mean fatty acid composition of the kephalin and lecithin have been reported (Gray, 1960). The fatty acid composition of the plasmal-free lecithins separated from the first three lecithin fractions is shown in Table 3.

Table 2. Fractionation of liver ester phosphatides L₃C on silicic acid

The starting material was the ester-phosphatide fraction C from a methanol extract L₃ after removal of insoluble barium salts of acidic phospholipids.

Eluate nos.	Fraction	Per cent of total P recovered	Molar ratio aldehyde:P (P = 100)	Double bonds/mol. of P
14-36	Kephalin	16	18	2.0
37-54	Lysokephalin	4	—	—
	Lecithin	5	—	—
55-67	Lecithin I	30	5	4.5
68-79	Lecithin II	32	11	3.0
80-91	Lecithin III	10	12	2.1
92-94	Lecithin IV	3	24	1.9

Table 3. Fatty acid composition of phospholipid fractions from ox liver

Gas chromatography of methyl esters on Reoplex 400 or Apiezon L. Values are expressed as percentage of the total methyl esters. tr., Trace.

Acids	Designation	Cardiolipin		Phosphatidylinositol		Lecithin L ₃ C		
		L ₄ A ₇ S	L ₄ A ₈ S	L ₂ A ₂ III	L ₄ A ₉	I	II	III
C ₁₀ -C ₁₈	—	—	0.8	0.5	—	tr.	0.2	2.4
Palmitic	16:0	0.9	1.7	1.9	1.9	8.3	15.3	18.7
Palmitoleic	16:1	1.3	1.5	—	0.3	—	0.6	2.2
Heptadecanoic	17:0	1.2	—	0.9	0.6	0.5	0.8	0.8
—	17:?	—	0.4	0.2	—	0.4	0.7	1.1
Stearic	18:0	8.4	1.8	54.6	46.8	39.6	35.0	25.6
Oleic	18:1	5.7	8.1	5.4	10.2	3.7	19.5	31.0
isoOleic	iso 18:1	—	—	2.6	—	3.7	—	—
Linoleic	18:2	63.0	70.0	3.5	8.6	2.9	11.3	11.6
Linolenic	18:3	12.0	15.5		2.5	tr.	tr.	—
Nonadecanoic	19:0	—	—	—	0.5	—	—	—
(Unidentified)	?	—	—	—	2.5	—	—	—
(Unidentified)	?	—	—	—	1.9	—	—	—
Eicosatrienoic	20:3	2.4	—	8.2	6.0	5.2	2.7	1.0
Arachidonic	20:4	2.8	—	16.8	7.1	11.4	5.3	3.2
Eicosapentaenoic	20:5	2.0	—	—	4.3	3.8	3.0	
Docosapentaenoic	22:5	—	—	5.2	7.1	17.5	5.1	1.4
Docosahexaenoic	22:6	—	—	—	—	2.9	0.3	0.8

DISCUSSION

Faure *et al.* (1959) found that the polyglycero-phosphatide from ox liver was identical with cardiolipin from heart muscle in general composition and serological activity. The chromatographic properties of the polyglycerophosphatide isolated in the present work, and the structure of the water-soluble ester prepared from it, confirm this identification. The discrepancies in earlier analyses (McKibbin & Taylor, 1952; Dawson, 1958; Marinetti, Erbland, Albrecht & Stotz, 1957) probably arise from the fact that the cardiolipin fraction obtained chromatographically is liable to be contaminated on the one hand with P-free lipid and on the other with degraded phosphatides. The Feulgen reaction given by crude preparations is attributable to rancidity rather than to the presence of plasmalogen derivatives, and it occurred whether the fractionation was carried out by chromatography or by solvent fractionation of the free acids. Cardiolipin and phosphatidyl-

inositol as well as the plasmalogens and ester phosphatides were substantially recovered from silicic acid columns, though prompt neutralization of acid solutions is desirable, but the possibility that some breakdown occurs either on columns or during solvent fractionation cannot be excluded. Because the proportion of cardiolipin is small the rancid or degraded phosphatide contaminating this fraction is possibly more noticeable in extracts of liver than from other tissues; it amounted at most to 0.4% of the total P, which is perhaps surprisingly low, considering the high degree of unsaturation of the liver phosphatides. Its accumulation in the fraction in which phosphatidic acid might be expected obviously complicates the unequivocal isolation of naturally occurring phosphatidic acid. The occurrence of small amounts of P-free lipid is probably another sign of breakdown. No evidence, however, was obtained of the presence of any substantial amount of phosphatidic acid or unidentified phospholipids in the early stages of fractionation.

In summary, cardiolipin has been isolated from ox liver by Faure *et al.* (1959) and, in the present work, in yields of 1–2% of the total phospholipid P; it has also been identified in rat liver (Macfarlane, Gray & Wheeldon, 1960) and in sheep liver (Dawson, 1960) in amounts approx. 3–4% of the total phospholipid. It is eluted from silicic acid columns with low concentrations of methanol in chloroform. The apparent failure of Hübscher & Clark (1960) to find any cardiolipin in ox or rat liver, fractionated by this method, and their success in readily isolating phosphatidic acid with 80–90% of unsaturated acids in yields of 0.8 and 1.5% of the total phospholipid, when other workers (e.g. Hokin & Hokin, 1958; Dawson, 1954, 1960; Macfarlane *et al.* 1960) have found phosphatidic acid in tissues only in trace amounts, if at all, call for some comments on their criteria of identification. (1) No evidence was offered, e.g. by paper chromatography of the intact phospholipid, that the nitrogen-free component was homogeneous and different from cardiolipin. (2) The main analytical difference between phosphatidic acid and cardiolipin is the ratio of glycerol to phosphorus. Hübscher & Clark (1960) apparently determined glycerol by periodate oxidation and estimation of formaldehyde after acid hydrolysis of the phospholipid. On acid hydrolysis cardiolipin gives firstly a high proportion of glycerodiphosphate (Gray & Macfarlane, 1958; Macfarlane & Wheeldon, 1959), which is not oxidized by periodate and only slowly hydrolysed in acid. No evidence is given that the conversion into orthophosphate and therefore the liberation of glycerol were complete. (3) The identification of 'glycerophosphate' formed from the phospholipid by mild alkaline hydrolysis was not rigorous, and no evidence was offered that the 'glycerophosphate' was distinct from the polyglycerophosphate derived from cardiolipin.

It should be emphasized that this criticism is not intended as an argument against the presence of naturally occurring phosphatidic acid, for if this substance is an intermediate it is very likely to be present. But the fact that Hübscher & Clark (1960) apparently did not find cardiolipin in the liver and have not, in the writer's opinion, excluded the presence of cardiolipin in their 'phosphatidic acid' fraction, together with the similarity of the fatty acid composition to that of cardiolipin, calls for some caution in accepting the isolated phosphatidic acid as the naturally occurring intermediate. As the authors themselves point out, it is difficult to reconcile the fatty acid composition with that of an intermediate in the biosynthesis of kephalin, lecithin or inositides, in which, generally speaking, 50% of the fatty acids are saturated.

In considering the fatty acid composition of the

cardiolipin and phosphatidylinositol isolated in the present work it should be noted that the degree of purity of the samples analysed was sacrificed in favour of a larger yield; the presence and effect of kephalin derivatives, the most usual impurity, can be assessed. The possibility that the apparent occurrence of stearic acid as the sole saturated acid in phosphatidylinositol might be due to selective isolation of a sub-family was mentioned previously (Gray & Macfarlane, 1958); the yield was here increased to 64–70% with 90% purity, and stearic acid is still clearly the predominant saturated acid; in samples from different animals the relative proportions of the different polyenoic acids however varied appreciably. The presence of 8% of stearic acid and polyenoic acids in the cardiolipin sample L₄A₈S can be correlated with the presence of 10% of kephalin indicated by the amino-N content. The predominant acid, as in cardiolipin from heart muscle, is linoleic acid, but linolenic acid was present in amounts (12–15%) considerably greater than were found in any other phospholipid.

The nature and structure of the unsaturated acids present in ox-liver ester phosphatides were established by Klenk and his colleagues (Klenk & Dreike, 1955; Klenk & Tomuschat, 1957). The mean composition of the ox-liver kephalin and lecithin fractions analysed by gas chromatography (Gray, 1960) correspond closely, qualitatively and quantitatively, with their findings; in this species, in contrast with the rat (Macfarlane *et al.* 1960), the C₂₂ pentaenoic acid preponderates over the C₂₂ hexaenoic acid.

The successive lecithin fractions obtained by chromatography on silicic acid (Table 3) had decreasing iodine values, due to increasing proportions of linoleic acid and oleic acid in place of higher polyenoic acids (cf. Rhodes & Lea, 1956). All the fractions contained the same proportion of saturated acids, with a higher proportion of stearic acid, particularly in the initial fraction eluted (I). Chromatographic fractionation of liver lecithin and hydrolysis with *Clostridium welchii* lecithinase could afford a useful range of diglycerides for experiments in biosynthesis.

SUMMARY

1. The polyglycerophosphatide of ox liver isolated by chromatography on silicic acid and by solvent fractionation of barium salts was identified as cardiolipin by the similarity in chromatographic and chemical properties of the phospholipids and of the phosphoric esters derived from them by mild alkaline hydrolysis.

2. The Feulgen reaction given by crude cardiolipin fractions is attributed to the presence of

rancid phosphatides, whose amount is small in relation to the total phospholipid.

3. Degraded phosphatides were present in the fraction expected to contain phosphatidic acid. It was therefore considered impossible either to exclude or confirm the presence of naturally occurring phosphatidic acid.

4. The bearing of these facts on the isolation of phosphatidic acid reported by Hübscher & Clark (1960) is discussed.

5. Linoleic acid (70 %) and linolenic acid (12–15 %) were the main fatty acids in ox-liver cardiolipin.

6. Phosphatidylinositol isolated in yields of 64–70 % of the total inositide contained stearic acid as virtually the only saturated acid (45–55 %) and polyenoic acids in varying proportion as the main unsaturated components.

7. Successive fractions of liver lecithin with decreasing iodine values obtained by chromatography on silicic acid contained similar proportions of saturated acids and decreasing proportions of polyenoic acids.

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The Amide Nitrogen Content of Gelatins

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It has long been known that the isoionic points of gelatins prepared from alkali-treated collagens are in general considerably lower than those of gelatins extracted after acid pretreatment. Ames (1952) has shown that this fall in the isoionic point of alkali-processed gelatins is associated with a loss of ammonia during pretreatment. The ammonia has been assumed to be derived mainly from hydrolysis of amide side chains which thus give rise to new free carboxyl groups in the intact molecule of the gelatin precursor. Even acid pretreatment may result in some loss of amide groups, if conditions are sufficiently severe (Ames, 1957). A picture has gradually emerged of a series of

possible gelatins, ranging from those with an isoionic point above pH 9, with substantially all the original amide groups still intact, to those prepared after a prolonged lime soak with a limiting minimum isoionic point at approximately pH 4.80 and presumably zero amide content. The exact relationship between the isoionic point and the amide content has not, however, been experimentally investigated. A survey of these two characteristics for a representative selection from a wide range of gelatins available in this Laboratory is described in this paper. For this purpose a rapid and accurate method for the determination of amide nitrogen in gelatin was developed.