utilizing relatively large amounts of acetate, must depend on a large and continuous supply of reduced pyridine nucleotide in order to promote lipogenesis and suppress ketogenesis. If the supply of glucose were reduced, or its breakdown retarded, the balance precariously maintained might easily be shifted in favour of ketogenesis. It thus seems not improbable that at least part of the acetoacetate found in the milk of ketotic cows (Robertson & Thin, 1953) originates in the udder itself, instead of being derived entirely from the liver and rumen. To those two important sites of ketogenesis the udder may possibly therefore be added as a third.

SUMMARY

1. Conditions affecting the balance between ketogenesis and lipogenesis in actively respiring homogenates of the lactating mammary gland of the guinea pig have been studied.

2. [carboxy- ^{14}C]Acetate and [2- ^{14}C]pyruvate, when incubated with mammary homogenates in the absence of added fumarate, were oxidized to carbon dioxide and converted into acetoacetate at comparable rates. The addition of fumarate in increasing amounts (1–10 mM), which, as previously shown (Terner, 1956*b*), results in the progressive stimulation of fatty acid synthesis, caused a progressive inhibition of acetoacetate formation.

3. When unlabelled glucose was added to mammary homogenates metabolizing [carboxy- ^{14}C]acetate in the absence of fumarate, it did not suppress and in some experiments promoted the incorporation of acetate carbon into acetoacetate. In the presence of both glucose and fumarate the formation of acetoacetate was abolished.

4. In marked contrast with the rapid rate of acetoacetate formation from acetate and from pyruvate, [^{14}C]glucose (randomly labelled) or [2- ^{14}C]lactate gave rise to only small amounts of the ketone body. It is suggested that this difference

resides in the ability of glucose and lactate to regenerate reduced pyridine nucleotide. The ability to maintain, in a vigorously oxidative system, conditions favourable for reductive reactions may be a common factor underlying not only the effects of fumarate on ketogenesis and lipogenesis in the present system, but also the well-known 'antiketogenic' properties of glucose and its stimulant action on lipogenesis.

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Separation and Composition of the Phospholipids of Ox Heart

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This work arose from a decision made in 1953 to prepare the acetal phospholipid described by Feulgen & Bersin (1939) and examine its susceptibility to various bacterial toxins. This acetalphosphatide, which at that time was regarded as a natural substance, is an acetal of a long-chain aliphatic aldehyde with glycerophosphorylethanolamine (I) from which aldehyde is

liberated on treatment with acid or mercuric chloride. It was presumed that this compound, with only one fatty chain, could be readily separated from the classical ester phosphatide by chromatography. However, Schmidt, Ottenstein & Bessmann (1953) suggested from the results of hydrolysis of crude brain-lipid extracts that the natural 'plasmalogen' was a compound of

glycerophosphorylethanolamine acetal with other alkali-labile lipid groups. Shortly after the present work was begun Klenk & Debuch (1954) obtained substantial evidence that the natural ethanolamine plasmalogen of brain contained a fatty acid as well as a fatty aldehyde, and they suggested as probable the structure (II) or (III) in which the aldehyde was placed arbitrarily on the α -carbon atom of the glycerol. These authors predicted that, after

(I)

(II)

(III)

treatment of the natural compound with dilute acid to remove the aldehyde, lysokephalin would be obtained. This evidence was obtained by Debuch (1956), after the isolation by Klenk & Debuch (1955), on an analogous prediction, of lysolecithin as a product of the mild acid hydrolysis of the plasmalogen-containing lecithin fraction of ox-heart muscle.

The work of Klenk & Debuch (1954) provided sound evidence that the natural plasmalogens contain a fatty acid. The position was thus that the natural plasmalogens had never been isolated, that they existed as choline, ethanolamine and serine derivatives and that their intimate structure was unknown. Evidence of the α -acyl- β -aldehyde structure (IV) of the ethanolamine and choline plasmalogens of ox heart (Gray, 1957*a*) and of the constituent aldehydes and fatty acids is given by

Gray (1958*a*). While this work was in progress, Rapport, Lerner, Alonzo & Franzl (1957) and Debuch (1957) showed that the linkage of the aliphatic residue with glycerol, which is broken by mild acid treatment, giving the free aldehyde, has the $\alpha\beta$ -unsaturated ether structure shown in (IV).

(IV)

The attempt described in this paper to prepare plasmalogens free from the classical ester phosphatides has been combined with a study of the nature and amounts of the phospholipids obtained from ox heart by two different methods of extraction and subsequently fractionated by chromatography on silicic acid. This method of fractionation, developed by Lea & Rhodes (1954*b*), had not been used with extracts containing acid-labile plasmalogens, and particular attention has been paid to the quantitative aspects and to the detection of minor components or of artifacts due to breakdown. Some data on the composition of cardiolipin and glycerophosphoinositide, and the use of a novel method for partition chromatography of phospholipids, are recorded.

Nomenclature. Feulgen & Bersin (1939), realizing that the acetal phospholipid (I) might be an artifact, suggested that the name 'plasmalogen' be retained for the natural lipid from which 'plasmal' (a mixture of aldehydes) was obtained by treatment with acid or mercuric chloride. In the intervening years this suggestion has been ignored and the names 'acetalphospholipid' and 'plasmalogen' used indifferently for natural and synthetic substances thought to be identical and now known to be different. Rapport & Alonzo (1955*b*) suggested the name 'phosphatidal choline' (or phosphatidal ethanolamine) for the plasmalogen compounds, but this word does not indicate the chemical structure, and its similarity to 'phosphatidylcholine' (lecithin) is confusing to the eye and ear. We have used 'plasmalogen' as a general name for the substances analogous to structure (IV); choline, ethanolamine and serine plasmalogen for the compounds characterized by the nitrogenous base; kephalin plasmalogen for fractions containing amino nitrogen; and lysoplasmalogen for compounds obtained by removal of the fatty acid from structure (IV). Removal of the aldehyde group from (IV) gives a lysolecithin or lysokephalin.

EXPERIMENTAL

Analytical methods

Aldehyde. The colorimetric method of Leupold & Buttner (1953) was used, with some adaptation. This is a two-stage reaction, the combined aldehyde being first split off in acid, and then condensed with the fuchsin reagent; this allows assessment of the acid-lability of plasmal compounds. Reagents: (i) 1.0 g. of rosaniline hydrochloride in 700 ml. of boiling water, filtered after cooling, 5.0 g. of $\text{Na}_2\text{S}_2\text{O}_5$ and 100 ml. of N-HCl , made up to 1 l., decolorized for 48 hr., stored at 2° in a dark glass-stoppered bottle with narrow neck. (ii) sulphite water (0.5% $\text{Na}_2\text{S}_2\text{O}_5$ in 0.1 N-HCl); (iii) Hopkin and Williams Ltd., 'capryl alcohol (octan-2-ol) "low in ketones" grade' was used for extraction of the coloured complex, as various brands of 'amyl alcohol' examined gave a high blank. For total aldehyde the lipid samples, together with two standards (10 and 20 μg . of palmitaldehyde as dimethylacetal) and a blank, were placed in glass-stoppered centrifuge tubes and the solvent was completely removed on a rotary evaporator. Acetic acid (0.5 ml. of 90%, v/v) was added, dissolving the lipid, and the tubes were kept at 50° for 45 min. Fuchsin reagent (2.0 ml.) was then added and after 20 min. at room temp. 2.0 ml. of sulphite water and 5.0 ml. of capryl alcohol were added. The tubes were shaken vigorously for 30 sec. and centrifuged for 4 min. The optical density of the coloured layer was measured in a Unicam spectrophotometer SP. 600 at 546 $m\mu$ or usually in an EEL colorimeter with filter 624. Free aldehyde, condensed in 90% acetic acid with ice-cold reagent at 2° for 30 min., gives 96% of the value obtained at room temp. but the dimethylacetal and native plasmalogen without previous hydrolysis give at 2° only 2-3% of the maximum.

The dimethylacetal of palmitaldehyde was used as standard. The method of Stephen (1925) for synthesis of palmitaldehyde failed (cf. Knight & Zook, 1953), but the method of Weygand, Eberhardt, Linden, Schafer & Eigen (1953), starting with palmitoyl chloride, gave palmitaldehyde in 85% yield. [The palmitoyl chloride was stated by the manufacturers, probably justifiably by classical criteria, to be 98% pure. Gas-liquid chromatography (see Gray, 1958a) showed that the aldehyde was a mixture of very closely related aldehydes, possibly including branched-chain forms. Apparently, 'pure' natural palmitic acid may be inhomogeneous.] The palmitaldehyde was refluxed with anhydrous methanol and benzene and a trace of toluene-*p*-sulphonic acid for 3 hr. and the water removed by azeotropic distillation. The dimethylacetal was isolated and redistilled twice *in vacuo*, giving an odourless, colourless liquid which solidified on storing at 2°. A stock standard was prepared by dissolving 48 mg. in 20.0 ml. of chloroform, and a working standard equivalent to 100 μg . of palmitaldehyde/ml. prepared by dilution.

The standard curve was linear in the range 5-50 μg . of aldehyde. On the basis of many readings with several batches of reagent, 10 μg . of palmitaldehyde in the test conditions gave a mean reading of $13.5 \pm 4\%$ EEL divisions with filter 624, or an extinction of 0.17 in a 1 cm. cell at 546 $m\mu$ with reagent decolorized at least 48 hr. The readings became higher as the reagent aged with loss of SO_2 ; standards to which the test samples were referred were always included in the test, and the reagent was dis-

carded when the normal colour value was significantly exceeded.

If the test sample contains much neutral fat, the mixture with the fuchsin reagent may be opalescent and a small amount of coloured solid may be left at the interface on extraction.

Comparison of the dimethylacetals of synthetic and natural aldehydes. As the colorimetric estimations were carried out on fractions containing a mixture of aldehydes and other phospholipids which might affect the result, the validity of the test was checked. A mixed lecithin-choline plasmalogen fraction contained by colorimetric assay 1.67 g. of aldehyde. The aldehydes were isolated quantitatively as their dimethylacetals (Klenk & Friedrichs, 1952); the yield was 1.85 g., equivalent to 1.62 g. of aldehyde by weight, and by colorimetric assay the product contained 1.59 g. of aldehyde. The colorimetric method therefore was a valid assay of the aldehyde content of the fractions. Since natural plasmalogens contain a mixture of aldehydes with differing colour values, results referred to a particular standard may not of course be absolutely correct.

Expression of results. As the molar ratio of P to aldehyde in plasmalogen is unity, the ratio moles of aldehyde/moles of P $\times 100$ gives the P present as plasmalogen as a percentage of the total P in a fraction; this was called the plasmalogen value.

Amino nitrogen. The colorimetric method of Lea & Rhodes (1954a) was used, and the optical density measured in the EEL colorimeter with filter 626 with recrystallized ethanolamine hydrochloride as standard (0.7 and 1.4 μg . of N). The ninhydrin reagent was unstable in spite of precautions to exclude light and oxygen and was made up on the day required. The colour value for serine/ μg . of N was 50% higher than for ethanolamine, but as in most of the fractions serine was present in very small proportion the method gave in general a reliable estimate of the ethanolamine nitrogen.

Fatty acid ester groups. The method of Rapport & Alonzo (1955a) was satisfactory, the optical density being compared in the EEL colorimeter, filter 624, with standards containing 1 and 2 μmoles of fatty acid ester. The colour yield with triolein and methyl palmitate increased slightly and that with lecithin decreased sharply if the time of heating at 65° with the alkaline hydroxylamine solution exceeded 60 sec. The colour yield also varies with the fatty acid present, or possibly with the complexity of the molecule; lecithin, lysolecithin, cephalin and lysocephalin gave 80-85% of the value with triolein or methyl palmitate per μmole of ester. A purified lecithin was therefore used as standard in analysis of phospholipid fractions, for which the method, though clearly not absolutely correct, is very useful.

Alkali isomerization of fatty acids. The method of Herb & Riemenschneider (1953) was adapted: 0.2 ml. (3-4 mg.) of fatty acids in methanol (0.2 ml. of methanol alone for the blank) was mixed rapidly with 3.0 ml. of 21% (w/w) KOH in redistilled ethylene glycol in glass-stoppered tubes which had been preheated 15 min. in a bath at 180°. The tubes were kept at 180° for 15 min. and then immediately cooled in water; the contents were transferred to a flask with methanol and made up to 25 ml. A further dilution (1:10) was read against the blank in a 1 cm. cell in the Uvispek spectrophotometer at 233, 268, 315 and 346 $m\mu$. The results were calculated according to formula (a) of

Herb & Riemenschneider and ascribed to linoleic acid, linolenic acid, arachidonic acid and C_{20-22} polyenoic acids. The acids were not formally identified and authentic standards were not available; the results, however, agreed well with gas-chromatographic analyses carried out by Dr A. T. James and Miss J. Webb at the National Institute of Medical Research, Mill Hill, London, N.W. 7.

Phosphomonoester. Identification of compounds as phosphoric monoesters, $R \cdot PO_3H_2$, was made by finding the proportion of P hydrolysed at pH 8.6 in 30 min. at 38° by a bone phosphatase free from diesterase, added in amount sufficient to hydrolyse any monoester in these conditions.

Choline phosphatides. Phosphatidylcholine, choline plasmalogen and sphingomyelin are hydrolysed by *Clostridium welchii* lecithinase. The amount of P present as these compounds was ascertained by estimation of the amount of acid-soluble P formed in 30 min. at 38° and pH 7.4 in borate buffer containing 0.004M-CaCl₂ in presence of excess of *Cl. welchii* lecithinase.

Inositol. Duplicate samples of lipid were hydrolysed in sealed ampoules in 6N-HCl for 41 hr. at 115°, adjusted to pH 5.1 and diluted for microbiological assay by comparison with dose-response curves with known amounts of inositol. We are indebted to Dr T. B. Bright of The Distillers Co. Laboratories, Epsom, for a culture of *Saccharomyces carlsbergensis* and details of the culture medium. The results were reproducible within about $\pm 10\%$.

Other methods. Estimations of phosphorus (Martland & Robison, 1926), total N (micro-Kjeldahl with Se catalyst), ethanolamine and serine (Axelrod, Reichenthal & Brodie, 1953), choline (Appleton, La Du, Levy, Steele & Brodie, 1953), glycerol (Blix, 1937), glycerolphosphate (Burmester, 1946), iodine value (Trappe, 1938), cholesterol (Rose, Schattner & Exton, 1941) and methylglyoxal (Ariyama, 1928) were carried out by standard methods.

Solvents and general precautions. Chloroform (B.P.), methanol and anaesthetic ether were used as supplied for extraction, fractionation and most of the chromatography. For analytical work or solution of fractions ether was dried with Na wire, and ethanol was freed from esters by keeping over NaOH and redistilling. Lipid fractions were never kept solid; solutions were concentrated under reduced pressure in a stream of N₂, usually not above 35° bath temperature; the residual solvent was removed *in vacuo* at room temp. and the lipid immediately redissolved in the required solvent. Washed extracts or fractions were usually kept in chloroform containing 2% of methanol, and were dried with anhydrous Na₂SO₄ or CaSO₄ before storage under N₂ at -10° in glass-stoppered flasks, and in these conditions were remarkably stable.

EXTRACTION AND FRACTIONATION OF PHOSPHOLIPIDS FROM HEART MUSCLE

Fractionation of phospholipids extracted with chloroform-methanol mixtures

The extraction was based on that of Folch, Ascoli, Lees, Meath & LeBaron (1951), but the amount of solvent was reduced for larger-scale work. Lean muscle from ox heart, obtained 2-3 hr. after death, was minced in a domestic mincer, and then blended in weighed portions with ice-cold solvent for 30 sec. in an M.S.E. blender at room temp. and kept 15-20 min. in large jars with occasional stirring. The extract was filtered on Büchner funnels, and the

residue was re-extracted. Four preparations were made by this method from 1.5 to 2.5 kg. of muscle and the following solvents: O₁, 2 vol. (l./kg.) of chloroform-methanol (2:1, v/v), re-extracted twice with 1 vol. of solvent; O₂, O₃ and O₄, 5 vol. of chloroform-methanol (1:1, v/v), re-extracted twice with 1 vol. of chloroform-methanol (2:1, v/v). Extracts O₁-O₃ were washed by stirring with 3-4 vol. of tap water; after 1-2 hr. the water was siphoned off, the voluminous white solid at the interface removed and drained, and the washing repeated, usually twice with tap water and once with distilled water at 2° overnight, till the volume of chloroform was constant. Extract O₄ was washed three times with 0.5% NaCl. The deep-yellow chloroform layer was put at -10° overnight to freeze the remaining water and filtered at 2°, and the solution dried over CaSO₄.

The yield and composition of the washed lipid, which included neutral fat and cholesterol, was substantially the same in the four preparations: 26-28 g. total wt., 570-600 mg. of P (about 14-15 g. of phospholipid)/kg. of fresh mince; molar ratio N:P between 0.9 and 1.1; amino N 37-42% of total N; plasmalogen value, 40-45%. Extract O₄ contained about 6% of the total N as serine, and 36% as ethanolamine; no inositol was detected. Extract O₁ contained 93-95% of the P as ester-P, estimated by the method of Schmidt, Benotti, Hershman & Thannhauser (1946), indicating that not more than 7% was present as sphingomyelin.

In a small-scale quantitative experiment, 10 g. of finely minced heart muscle was extracted with 20 vol. of chloroform-methanol (2:1, v/v) for 2 hr. at room temp. and washed as described by Folch *et al.* (1951); the chloroform solution contained 5.6 mg. of P (N:P ratio 1.1) and the interfacial solid contained 0.55 mg. of P (N:P ratio 3.9). The yield of phospholipid obtained in the large scale agrees with that in the small scale, and broadly with amounts found in heart muscle of various species in quantitative studies.

Separation of neutral fat from phospholipid. The phospholipid was separated from neutral fat by passing the solution through silicic acid with chloroform (Borgström, 1952). The phospholipids are quantitatively adsorbed, and subsequently eluted with methanol. The phospholipid was then rechromatographed on silicic acid (Lea & Rhodes, 1954b; Lea, Rhodes & Stoll, 1955) with chloroform with increasing concentrations of methanol, which displaces the phospholipids in the order phosphatidic acid, kephalin, lysokephalin, lecithin and lysolecithin.

Silicic acid prepared by the method of Isherwood (1946) was variable in properties. Silicic acid (Mallinkrodt A. R.) has been used throughout; the particle size is regular (100-mesh) and the flow rate was suitable without the use of a filter aid. The solvent used for elution was adjusted according to the properties of a particular batch of silicic acid within the range methanol-chloroform 1:9 to 1:4 for kephalin and 2:3 to 3:2 for lecithin. This silicic acid is, however, apparently so active that, in certain circumstances which are not fully under-

stood, breakdown of plasmalogens can occur on the column. This is shown in the following experiment in which identical portions of a lipid extract were run in succession through the same column, breakdown occurring in the first but not in the second run.

The washed lipid extract O_2 was divided into equal portions. The first portion, O_2a (385 mg. of P), in 250 ml. of chloroform was loaded on a column (diam. 40 mm.; 230 g. of silicic acid) and chloroform was passed under pressure (30–40 mm. Hg) of N_2 until the neutral fat was washed through; this required 1.75 l. and about 15 hr. The phospholipid, visible as a series of brown bands, was then eluted with chloroform-methanol (1:3, v/v) (2.5 l.; 30 hr.). The column was washed with chloroform till free from methanol and percolated overnight. The second portion of lipid extract O_2b was then chromatographed similarly. Analysis of the fractions from both runs showed an excellent recovery of P (97 %) in the phospholipid fractions, but 36 % of the plasmalogens in O_2a had been split, giving free aldehyde which appeared in the fat fraction, and presumably lysophosphatides. In the second run, O_2b , the plasmalogens originally present were recovered in the phospholipid fraction. Part of the cholesterol was retained with the phospholipid. A small amount of P ran in the fat fraction in the tail of the main cholesterol fraction.

The only difference in the two runs was that the silicic acid had been aged by the first run. It has been found in many subsequent runs that in the absence of the fat fraction the plasmalogens are stable on the silicic acid columns, and they are also stable for 14 days or so in chloroform solution containing the fat. It seems possible that there can be a reaction between very active silicic acid and some component of the fat which increases the acidity of the column sufficiently to decompose the acid-labile plasmalogens.

Fractionation of phospholipids on silicic acid.

(i) A portion of phospholipid fraction O_2a (145 mg. of P) which presumably contained lysophosphatide derived from plasmalogens, was chromatographed on a column (diam. 31 mm., height 280 mm.)

containing 100 g. of silicic acid in methanol-chloroform (1:4, v/v). The kephalin band was eluted with this solvent and elution continued with methanol-chloroform (1:1) until the lecithin band was off. The separation curve based on analyses of 15 ml. fractions for P, amino N and aldehyde is shown in Fig. 1. A second portion of O_2a chromatographed in the same way gave an almost identical curve. The fractions from both runs were pooled on the basis of the curves into four fractions (approximately phosphatidic acids, kephalin, lyso-kephalin and choline phosphatide) A, B, C and D, the analyses of which are given in Table 1.

(ii) A portion of phospholipid fraction O_2b (66 mg. of P), in which the plasmalogens were intact, was chromatographed by the same procedure on 40 g. of silicic acid. The separation curve (8 ml. fractions) is shown in Fig. 2. The fractions were pooled into four main fractions A, B, C and D as before (Table 1).

In both runs, the front band A, containing 12–15 % of the total P and a little cholesterol, was dark brown and low in N, and was presumed to be mainly cardiolipin (see p. 420). The kephalin fraction B, brownish yellow, contained 24 % of the

Fig. 1. Fractionation of phospholipid extract O_2a on silicic acid. ○, Total P; ●, plasmalogen P, calc. from aldehyde content; △, amino N.

Table 1. Separation of components of the phospholipid preparations O_2a and O_2b

O_2a : 290 mg. of P; recovered from column, 278 mg. O_2b : 66 mg. of P; recovered from column, 62 mg.

Component	Fractions from O_2a				Fractions from O_2b			
	A	B	C	D	A	B	C	D
P (mg.)	33	67	78	100	9	22	11	20
N (mg.)	4	29	39	48	0.5	10	5	10
Amino N (mg.)	2	28	23	2	0.6	11	1	0
Aldehyde (mg.)	27	80	131	445	5	82	32	109
P (% of recovered P)	12	24	28	36	14	36	18	32
% of P in fraction as kephalin	13	90	60	4	14	100	20	Nil
% of P in fraction as plasmalogen	10	15	20	55	6	45	36	67

total P in O_2a , against 36 % in O_2b , and the plasmalogen value was 15, against 45 %, a decrease equivalent to 12 % of the total P. The lysokephalin content of fraction *C* was calculated from the ratio amino N/total N; from this, the percentage of the total P present as lysokephalin was estimated as 16 % in O_2a and 4 % in O_2b . The loss of kephalin plasmalogen in O_2a was therefore almost exactly balanced by the appearance of lysokephalin. The colourless fraction *D* contained no amino N; the total amount of choline phosphatides, a mixture of lecithin and choline plasmalogen, calculated as the sum of *C* and *D*, minus lysokephalin, was equivalent to 48 % of the total P in O_2a and 46 % in O_2b , with plasmalogen values of 56 and 61 % respectively, showing that there had been little, if any, breakdown of choline plasmalogen. About 95 % of the P was recovered. The sphingomyelin band was not eluted.

If the amino N in fraction *C* is regarded as lysokephalin derived from the breakdown of ethanolamine plasmalogen, the distribution of the lipid P in the chloroform-methanol extract of heart was approximately cardiolipin 12, kephalin (ester phosphatides) 22; ethanolamine plasmalogen, 20; lecithin, 21; choline plasmalogen 25 %.

Fractionation of phospholipids extracted with methanol followed by chloroform

Several extractions (O_5 – O_{10}) were made by the method of Pangborn (1945), i.e. extraction with methanol after dehydration and defatting with acetone, and recovery of the acidic phospholipids as the barium salts. About 80 mg. of lipid P/kg. of mince was lost in the acetone extracts. The amount of lipid P extracted with methanol was less than with chloroform-methanol; re-extraction

of the residue with chloroform yielded a further amount of phospholipid. The protocol of one experiment follows.

Protocol. O_{10} . Ox heart (7.25 kg.) was blended mechanically with the solvent and extracted twice with 1.2 l. of acetone/kg. The residue was extracted with 2 l. of methanol/kg. of mince in large bottles at 2° for 4 days, with occasional shaking, then filtered off and washed on the funnels with methanol. (The extraction of phospholipid was 94 % of the maximum in 24 hr.) The yellow extract (17 l.) was treated with aqueous 40 % $BaCl_2$, and the clear supernatant collected by decantation and centrifuging. The precipitated Ba salts, containing acidic phospholipids, nucleotides and inorganic phosphate, were quickly washed in 20 % (v/v) acetic acid, and the ether-soluble Ba salts separated into two fractions by dissolving three times in ether and reprecipitating with 2 vols. of methanol. The methanol-insoluble Ba salts were converted into Na salts (fraction *A*, Table 2; total P, 350 mg.); the methanol-soluble supernatant (fraction *B*) contained 120 mg. of P.

The main methanol extract, after removal of the insoluble Ba salts, was concentrated to 3 l.; an equal volume of chloroform was added, and the mixture washed three times with about an equal volume of water. The washed chloroform solution was concentrated and dried (fraction *C*, 2430 mg. of P).

The mince residue, after the extraction with methanol, was stirred with chloroform (about 700 ml./kg. of mince) and filtered off after a few hours at room temperature. The extract was highly pigmented and contained neutral fat. An equal volume of methanol was added, and the mixture washed with water. The washed chloroform solution was concentrated to about 400 ml. and poured into 4 vols. of acetone. The precipitated phospholipid was dissolved in chloroform, giving a brown solution (fraction *D*, 630 mg. of P); the acetone supernatant contained 42 g. of solids, but only 30 mg. of P.

Chromatographic fractionation of fractions C and D

Portions of fractions *C* and *D*, each containing about 120 mg. of P and a little fat, were chromatographed on silicic acid in identical columns by the usual procedure. The separation curves are shown in Fig. 3*a* and *b*. Fraction *C* separated into three distinct bands corresponding approximately to kephalin, lecithin and sphingomyelin. Fraction *D* gave three similar bands and two small fast-running bands. The eluate fractions were pooled as shown in Table 2, the front and rear portions of each of the kephalin and lecithin bands being kept separate. Each pool was concentrated and analysed without further purification. The distribution and analyses are shown in Table 2.

The total lipid P recovered in the fractions *A*–*D* was 490 mg./kg. of mince, which, with the additional amount lost in the acetone, is in good agreement with the chloroform-methanol extracts (p. 412). The methanol extract contained 82 % of the total P, but there was some differential extraction, as this extract contained about 95 % of the choline phosphatides, but only 70 % of the

Fig. 2. Fractionation of phospholipid extract O_4b on silicic acid. ○, Total P; ●, plasmalogen P, calc. from aldehyde content; △, amino N.

Table 2. *Distribution and analysis of phospholipids in fractions from methanol and chloroform extracts of ox heart*

		Analysis of fraction						
Fraction	P content as % of total lipid P	% P on dry wt.	Molar ratio (P = 100)				Double bonds/mol. of fatty acid	
			Total N	Amino N	Aldehyde	Fatty acid ester		
Methanol extract								
<i>A</i>	10.0	3.5	16	20	6	190	—	
<i>B</i>	3.0	—	—	97	30	—	—	
<i>C</i>	69.0	—	—	38	56	—	—	
Subfractions of <i>C</i>								
Tubes	Fraction							
35-41	3	5.0	3.7	99	90	33	167	1.99
42-61	4	18.4	4.1	96	100	58	152	1.91
71-84	5	4.7	3.6	106	16	37	162	2.02
85-120	6	36.6	4.0	99	Nil	59	148	1.21
121-165	7	4.2	3.3	214	Nil	Nil	Nil	—
Chloroform extract								
<i>D</i>	18.0	—	—	66	45	—	—	
Subfractions of <i>D</i>								
Tubes	Fraction							
10-31	1	2.2	4.2	4	12	17	190	1.64
32-38	2	1.6	3.5	—	25	6	185	1.93
39-51	3	8.5	3.8	91	100	43	165	1.86
52-85	4	3.2	3.8	87	100	46	150	1.43
86-96	5	0.1	—	—	66	14	—	—
97-124	6	1.8	3.8	98	Nil	51	148	1.13
125-176	7	0.5	2.7	101	Nil	Nil	100	—

kephalin phosphatide. The analyses (Table 2) indicate that the extraction into methanol is influenced by the nature of the fatty acids; calculated from the iodine uptake (after correction for the iodine uptake of the $\alpha\beta$ -unsaturated linkage of the aldehyde component) the kephalin and choline phosphatide fractions in the methanol extract had a higher degree of unsaturation than those in the chloroform extract.

Nature of the phospholipids in the various fractions (Table 2)

Acidic phospholipids. Fraction A, containing the acidic phospholipids with Ba salts insoluble in methanol, is a mixture containing about 20% of inositide and 16% of nitrogen-containing phosphatide, including almost all the detected serine phosphatide (serine N, 60% of total N in fraction A). The remainder of this fraction is presumably cardiolipin, but a satisfactory quantitative separation of the components present has not yet been achieved (see p. 421).

The chloroform extract D on chromatographic fractionation gave two small fast-moving bands (D_1 and D_2), low in amino nitrogen. Fraction D_2 was in the usual position for the cardiolipin band,

but it was thought that the fraction D_1 , eluted in chloroform alone, might be a simple phosphatidic acid, either a natural intermediate or an artifact formed by breakdown of lecithin or kephalin. The constituent fatty acids were therefore isolated from a portion of D_1 after alkaline saponification, as a pale-yellow oil, iodine no. 161; no solid acids separated at 2°; alkali isomerization indicated that 82% of the total acids were present as linoleic and linolenic acids. The water-soluble P ester obtained after saponification in methanolic 0.2N-NaOH for 15 min. at 37° gave only 45% of the P as glycerol phosphate after treatment with periodate at 100° in N-H₂SO₄ (cf. Table 7). No Na or K was detected in fractions D_1 and D_2 . It was concluded that D_1 was substantially cardiolipin, possibly in a 'polymerized' form (see p. 421).

Ester phosphatides. Fractions C_3 , C_4 , D_3 and D_4 (Table 2) are substantially mixtures of phosphatidylethanolamine and ethanolamine plasmalogen; the serine N was less than 10% of the total N in these fractions. The slightly low N content of D_3 and D_4 may indicate the presence of a nitrogen-free phosphatide in amounts equivalent to about 1% of the total lipid P. Fractions C and D are mixtures of phosphatidylcholine and choline

plasmalogen; all the P in these fractions was hydrolysable by *Cl. welchii* lecithinase.

Lysophosphatides. Lysokephalin moves with the front of the lecithin band. Fractions C_5 and D_5 contained amino N equivalent to about 0.8% of the total lipid extracted as lysokephalin. No evidence of the presence of lysolecithin was found, though it is impossible to exclude it absolutely.

Sphingomyelin. Fractions C_7 and D_7 , obtained from small peaks in the tail of the lecithin band, were substantially sphingomyelin; the ester P was only 11–14% of the total P. The low percentage of P on the dry wt., due to traces of silicic acid in the relatively large volume of solvent required for elution, was raised to 3.9% after precipitation with ether from chloroform solution.

Composition of ox-heart phospholipids

From the analyses in Table 2 the distribution of phospholipids was computed (as % of the total lipid P) as: inositide, 2; cardiolipin (or similar compounds), 9; serine derivatives, about 5; phosphatidylethanolamine, about 20; ethanolamine plasmalogen, 18; phosphatidylcholine, 18; choline plasmalogen, 24; sphingomyelin, 5%. The amounts are very similar to those obtained by fractionation of the chloroform-methanol extract (p. 414).

Fig. 3. Fractionation of ox-heart phospholipid on silicic acid. (a) Methanol extract, fraction C; (b) chloroform extract, fraction D. ○, Total P. The ringed numbers indicate the subfractions (Table 2).

The foregoing experiments show that fat-free phospholipid extracts containing plasmalogens can be chromatographed on silicic acid without breakdown and with good separation of the main kephalin- and choline-based components from each other. But, although the rear portions both of the kephalin and lecithin bands were richer in plasmalogens than were the front portions (see Fig. 1), the degree of separation was too small to be useful for the preparation of pure plasmalogens. A further attempt to separate plasmalogen from ester phosphatide on silicic acid was made by means of the gradient-elution technique developed by Lakshmanan & Leiberman (1954), but the enrichment was of no practical value. The non-polar groups of the phospholipids have clearly too small an influence on the degree of adsorption (cf. Lea *et al.* 1955) to permit separation of the ester and plasmalogen families from each other.

FRACTIONATION OF PHOSPHOLIPIDS BY PARTITION CHROMATOGRAPHY

The chances of separating a plasmalogen from the analogous ester phosphatide should be increased if the influence of the polar part of the molecule is decreased and the difference in the non-polar part is accentuated. Gray (1957*b*) pointed out that partition chromatography with purely organic solvents should be particularly useful for the separation of lipids and found that a secondary cellulose acetate, acetyl value 52.5%, was suitable as a support.

Preliminary experiments were carried out with a lecithin-choline plasmalogen fraction and several solvent systems to see (a) whether there was a significant difference in the partition function of choline plasmalogen and lecithin, (b) whether the stationary phase was a good solvent for phospholipids, and (c) whether the partition functions of these phospholipids varied with their concentration.

Quantities of the two solvents of each system were shaken together and the equilibrated phases were separated and used as the appropriate stationary or mobile phase. The phospholipid (about 1.0 mg. of P) was dissolved in 2.0 ml. of the equilibrated stationary phase and shaken for a set time with 2 ml. of the mobile phase. After separation of the layers the mobile phase was removed and analysed for P and aldehyde content. Sufficient of the stationary-phase solvent was added to keep the phase volume constant, and the procedure was repeated 5–10 times. The percentage of choline plasmalogen in both phases was calculated. The results were disappointing in that any difference in the partition of the two components was within the experimental error, i.e. less than 8% with the solvents tested.

The partition functions of the two compounds were so similar that it was possible to treat the phospholipid mixture as a single solute dispersed between the stationary- and mobile-phase solvents. The concentrations of the mixture in both phases of several solvent systems were plotted (Fig. 4a). The logarithmic values of the concentrations were also plotted and were found to be on a straight line (Fig. 4b). Therefore, the forms of the curves (distribution isotherm) were those covered by the Freundlich isotherm equation:

$$C_s = KC_m^n,$$

where C_s is the concentration of solute in the stationary phase, C_m the concentration of solute in the mobile phase, and K and n are constants. This showed that the partition function of the phospholipid solute varied with the concentration.

As even a 5% difference in partition function between the plasmalogen and the ester phosphatide might be enough for separation with solvent systems in which the R of both solutes is low, a number of partition columns were examined. A full account of these is given by Gray (1958b). The

secondary cellulose acetate used was a standard commercial product of British Celanese Ltd., grade CRIFS, acetyl value 52.5–53.5%, supplied as a powder (50-mesh). The phospholipids chromatographed were mixtures of lecithin and choline plasmalogen, similar to fraction C_6 , Table 2.

(i) *Solvent system propylene glycol-carbon tetrachloride*

This system gave promising results but was abandoned because some breakdown of plasmalogen occurred. This was traced to the presence in the propylene glycol of some impurity, possibly secondary halides, giving rise to HCl. Even after refluxing with 70% NaOH, fractional distillation and shaking with Bio-Deminrolit (The Permutit Co. Ltd., London, W. 4), the propylene glycol available when mixed with carbon tetrachloride gave free acid within 48 hr.

(ii) *Solvent system light petroleum-methanol*

Column 42. The equilibrated stationary (methanol) phase (100 ml.) was mixed with 80 g. of cellulose acetate and enough mobile phase was added to give a thin slurry, which was shaken for 1 hr. The column (20 mm. diam.) was built up to a height of 600 mm., a head of light petroleum (b.p. 60–80°) being kept above the stationary phase to prevent bubble formation. It was necessary to lag the column and solvent container, as a change in temperature disturbed the equilibrium; and several lamps were connected with a thermostat at $22 \pm 0.5^\circ$ round the apparatus. The column was tested for uniformity of packing by running through a mixture of Sudan III and Sudan IV dyes.

The phospholipid (1.25 mg. of P; plasmalogen value 54%) was chromatographed at a flow rate of 5.0 ml./hr. under positive nitrogen pressure of 50 mm. Hg. and fractions were collected every hour. Approximately three column volumes (190 ml.) of eluate were collected before phospholipids appeared in the fractions. The first two fractions, containing 4% of the total P, contained only 20% of plasmalogen. In the next few fractions the plasmalogen value rose, to as high as 90% in two tubes, and then gradually decreased to only 11%. The eluate fractions were pooled into five main fractions, A–E, which were analysed (Table 3). Fraction B, which contained 72% of choline plasmalogen, an increase of 18% on the original material, was rechromatographed on a small column (no. 43), diam. 11 mm., height 350 mm., flow rate 2.0 ml./hr.; 27% of the P was recovered in a fraction with a plasmalogen value of 86%. The overall yield was 9% of the total P in the starting material, which had a plasmalogen value of 54%, i.e. 14% of the plasmalogen P was recovered as 86% pure.

Fig. 4. Distribution of choline phosphatide between two immiscible organic phases. C_s = concentration of solute in stationary phase. C_m = concentration of solute in mobile phase. Phases (mobile first): $\circ$, triethylene glycol-carbon tetrachloride; $\bullet$, diethylene glycol-ethyl butyrate; Δ , propylene glycol-carbon tetrachloride; $\blacktriangle$, carbitol-hexane.

It is apparent that the *R* values of some of the lecithins are almost the same as those of the plasmalogens in this solvent system. The spread of the band in column 42 was greater than would be expected for the amount of separation actually obtained, but as the two compounds are presumably both present as families the spreading of the band could be due to fractionation of members of each family according to the constituent fatty acids. The tailing was probably caused by the fact that the partition function of a phospholipid solute between a two-phase solvent system (Fig. 4) varies with concentration; as the concentration of the phospholipid in the more polar solvent became less the ratio of the phospholipid in the polar phase to that in the non-polar phase became greater. Therefore as the concentration of phospholipids decreased more of the mobile phase was required to elute them, so that the tail, though containing very little of the total phospholipid, was extremely long.

(iii) *Solvent system cyclohexane-methanol*

In the light petroleum-methanol solvent system the *R* of the front fractions was approximately 0.33. The partitioning of the phospholipids was more in favour of the methanol in a *cyclohexane-methanol* system than in the light petroleum-methanol system, from which it was inferred that on a partition column the *R* values would be lower.

Column 45. A column (diameter 10 mm., height 380 mm.), containing 14.5 g. of cellulose acetate supporting the methanol stationary phase, was equilibrated with the *cyclohexane* mobile phase at 22°. Good lagging and temperature control were necessary. A lecithin-choline plasmalogen fraction (plasmalogen value 57%; 0.5 ml.; 1.24 mg. of P) was settled in and elution was begun with a flow rate from the column of 3.0 ml./hr. under a positive nitrogen pressure of 150 mm. Hg. Fractions were

collected every hour and analysed for P and aldehyde, and the separation curves for the choline plasmalogen and the lecithin plotted (Fig. 5). The *R* of the first phospholipid fractions was approximately 0.22. A good enrichment in choline plasmalogen was obtained in fractions 21-28, some fractions having a plasmalogen value of more than 90%. Fig. 5 shows a small band of phospholipid (fractions 15-19) with a low plasmalogen value running ahead of the main choline-plasmalogen fractions; no amino nitrogen was found in this band. It was thought most probable that the band contained a lecithin which, because of the nature of its fatty acids, was slightly less soluble in methanol than was the main lecithin fraction and which was therefore eluted a little in front of the other lecithins.

The eluate fractions were pooled to give seven main fractions. The results (Table 4) show a small improvement over those obtained with the light-petroleum-methanol system on column 42, which was a much longer column. A bigger column was used for fractionation of larger quantities.

Fig. 5. Partition chromatography of a lecithin-choline plasmalogen mixture on cellulose acetate. Column 45. Solvent system: *cyclohexane-methanol*. O, Choline plasmalogen P, calculated from the aldehyde content; ●, lecithin P, calculated as total P minus plasmalogen P.

Table 3. *Partition chromatography of a lecithin-choline plasmalogen mixture*

Column 42. Stationary phase, methanol. Mobile phase, light petroleum (b.p. 60-80°).

	Original material	Fractions				
		A	B	C	D	E
Plasmalogen value (%)	54	20	72	61	51	11
P in fraction (% of total)	100	4	34	39	4	19

Table 4. *Partition chromatography of a lecithin-choline plasmalogen mixture*

Column 45. Stationary phase, methanol. Mobile phase, *cyclohexane*. The mixture contained 57% of choline plasmalogen.

	Fractions						
	I	II	III	IV	V	VI	VII
Tubes	15-19	21-28	29-41	42-51	52-63	64-76	77-90
P as % of total P	2	28.5	33.8	16.9	7.6	7.6	3.6
% of P in fraction as plasmalogen	10	85	62.5	39	28	13.5	12.4

Table 5. *Iodine values of successive fractions of a lecithin-choline plasmalogen mixture obtained on a cellulose acetate partition column with cyclohexane-methanol*

	Fractions					
	A	B	C	D	E	F
P (μ moles)	72	216	110	88	52	13
Plasmalogen (μ moles)	58	127	59	41	23	5
Fatty acids (μ moles) calc.*	86	305	161	135	81	21
Iodine uptake (μ moles)	226	577	284	219	125	29
Iodine uptake of fatty acids (μ moles)†	168	450	225	178	102	24
Double bonds/fatty acid	1.95	1.47	1.40	1.32	1.26	1.13

* Calc. on 2 moles of fatty acid/mole of P for ester phosphatide, 1 mole/mole of P for plasmalogen. Ester phosphatide = μ moles of P - μ moles of plasmalogen.

† Corrected for I_2 uptake of aldehyde, 1 μ mole/ μ mole of plasmalogen.

Column 53. A column (diameter 39 mm., height 670 mm.), containing 238 g. of cellulose acetate supporting the methanol stationary phase, was equilibrated with the mobile phase. It was much more difficult to obtain a uniform and tight packing with the large column and slight distortion of the band fronts and more band-spreading occurred. Lecithin-choline plasmalogen (20 mg. of P; plasmalogen value 57%) was eluted at a flow rate of 20 ml./hr. under a positive nitrogen pressure of 50 mm. Hg. Fractions were collected every hour and analysed for P and aldehyde. There was a slightly lower degree of resolution of the lecithin and choline plasmalogen on the larger column; with the small column 45 the front fractions (fraction II, tubes 21-28) contained 28.5% of the total phospholipids with a plasmalogen value of 85%; with the large column 53 the corresponding fractions contained 20% of the total phospholipids with a plasmalogen value of 79%.

Effect of the constituent fatty acids on the fractionation. It was suggested above (p. 418) that the asymmetric shapes of the separation curves obtained (Fig. 5) were due to some separation of the individual lecithins and plasmalogens, owing to differences in the chain length and unsaturation of the constituent fatty acids.

The iodine values were estimated on the fractions from another fractionation of a lecithin-choline plasmalogen mixture with the partition system cyclohexane-methanol. The results (Table 5) showed that the front fractions contained more highly unsaturated acids than did the back fractions, indicating that the fatty acids in the plasmalogens were more unsaturated than those in the lecithins. This fact was confirmed later by examination of the fatty acids of pure lecithin and pure α -acyl- and β -acyl-lysocleithins prepared from a lecithin-choline plasmalogen mixture (Gray, 1958a).

A number of fractionations of phospholipid mixtures with a choline plasmalogen value of between 50 and 60% have been carried out on a

preparative scale with the large column. In general the method yielded approximately 20% of the total phospholipids as fractions with plasmalogen values of 75-85%. Mixtures of kephalin and ethanolamine plasmalogen were also fractionated on cellulose acetate columns with a cyclohexane-methanol solvent system. The results obtained were similar to those with the choline-containing mixtures, the front phospholipid fractions being enriched in plasmalogens whereas the rear fractions were richer in kephalins. The disadvantage of the cyclohexane-methanol system for the separation of kephalin mixtures was the low solubility of the phospholipid in methanol, which caused it to pass through the column much too quickly for a reasonable resolution to be accomplished.

Excellent separations were obtained when lecithin-lysocleithin mixtures were chromatographed on cellulose acetate with a cyclohexane-methanol system. The lecithins were eluted first, well separated from the lysocleithins, which were eluted very slowly in cyclohexane but could be rapidly recovered by passing methanol through the column.

Many variations of solvent systems are possible with these partition columns and some may give better separations than those so far tried. However, the cyclohexane-methanol system with cellulose acetate as support has been satisfactory for the preparation of plasmalogen-rich samples of phospholipid. The conditions on the columns are so mild that even a slight breakdown of a phospholipid is unlikely to occur, in contrast with systems in which the adsorbent is acid (silicic acid) or alkaline (alumina). The phospholipids can be quantitatively recovered, the column can be standardized and, provided that it is equilibrated after each fractionation, used repeatedly.

CARDIOLIPIN

Cardiolipin was isolated from ox heart by Pangborn (1942) and is used as a constituent of the Wasserman antigen in the diagnosis of syphilis;

a method for its preparation within certain limits of purity for this purpose has been prescribed (Pangborn, Almeida, Maltaner, Silverstein & Thompson, 1955), involving repeated precipitations of the barium, sodium and cadmium salts.

Cardiolipin was considered by Pangborn (1947) to be a glycerylphosphatide derivative containing three phosphoric acid molecules linking four glycerol molecules, the latter being esterified with six fatty acids, in the ratio five linoleic to one oleic residue. This constitution was suggested on the basis of the best fit of the analytical data for the original compound, and for the polyglycerophosphate obtained by mild saponification, and it has never been proved. A bar to further work is the difficulty of purification by the standard method described for the preparation of cardiolipin as a serological reagent, and attempts were therefore made to prepare cardiolipin chromatographically.

Preparation of cardiolipin

(a) *Chromatography on silicic acid.* (i) The front fractions, mainly kephalin, obtained by chromatography on silicic acid of a phospholipid extract (O_3 , p. 413) were re-run on a silicic acid column, beginning the elution with methanol-chloroform (1:9, v/v). Fig. 6 shows the separation of the presumed cardiolipin band (fraction A) from kephalin (fraction B). Fraction A was very dark-coloured, and was precipitated three times from concentrated solution in ether with acetone, which removed most of the pigment (Found: P, 4.0; N, 0.12; aldehyde, 0.6; glycerol, 17.8%; $[\alpha]_{5461}^{22} + 8^\circ$ in CHCl_3) (c, 3.7); molar ratio P:glycerol:fatty acid ester, 1:1.5:2. By gas-chromatographic analysis of

the methyl esters the fatty acids contained about 80% of linoleic + linolenic acids. This material contained no Na or K, and after storing some time became insoluble in ether; it was then serologically inactive, presumably due to some change in the free acid with time (cf. Pangborn, 1944). It was concluded that the material was cardiolipin, the only obvious discrepancy being a molar ratio of glycerol to P of 3:2, instead of 4:3 on Pangborn's structure.

(ii) A methanol extract of heart muscle (O_5), prepared by Pangborn's (1945) method (p. 414), was concentrated *in vacuo* and the phospholipid extracted into chloroform. After being washed with water and dried, the chloroform solution (P, 9; Na, 5.7; K, 2.8 m-moles) was chromatographed on silicic acid, elution being begun with methanol-chloroform (1:19, v/v). The dark-coloured front fraction (about 5% of the total P) was concentrated, and the phospholipid was precipitated from ether with acetone, removing most of the pigment, with some loss of P, and dissolved in ether. The solution contained P 258, amino-N 4, Na 4.3, K 0.6 μ moles. The Ba salt was immediately prepared by pouring the ethereal solution into 2 vol. of methanol containing 0.2 ml. of 20% aqueous BaCl_2 , and was reprecipitated from ethereal solution with methanol. It was converted into the Na salt by shaking the ethereal solution with 0.1 vol. of ethanol and 5% aqueous Na_2SO_4 ; the ethereal solution was dried and evaporated and the residue dissolved in dry ethanol. After 48 hr. at 2° the solution (O_5/b) was decanted. This material was serologically active.

(b) *Combination of fractional precipitation and chromatography.* The acidic phospholipids were precipitated as Ba salts from a methanol extract of heart muscle (O_7 , p. 414). The Ba salts were purified by washing with acetic acid, and reprecipitating several times from ethereal solution with methanol, and were converted into Na salts. The material (molar ratio P:amino N:inositol, 1:0.2:0.2) was dissolved in chloroform and chromatographed on silicic acid, beginning with methanol-chloroform (1:19, v/v). The front, fast-running band contained 8% of the total P and no Na or K. The middle fractions, $O_7/16$, contained 10% of kephalin and 10% of inositide and the rear fractions, $O_7/20$, about 50% of inositide. The fraction $O_7/16$ was rechromatographed on silicic acid; 50% of the P was recovered in the front fraction, free from inositide, but containing 10% of kephalin. This fraction was further purified by dissolving the salt in ethanol (about 1%) and decanting the solution ($O_7/16S$) after 48 hr. at 2° .

(c) *Method of Faure & Morelec-Coulon* (1956). The acidic phospholipids in a methanol extract (O_8) of heart muscle were separated and purified as the Ba salts. The free acids were obtained in ethereal solution by treatment with methanolic HCl and saline, and fractionated in concentrated ethereal solution by addition of ethanol; cardiolipin remained in solution, practically free from nitrogen and inositide. It was recovered as the Ba salt and converted into the Na salt. The ethanolic solution ($O_8/9S$) of the Na salt was decanted after 48 hr. at 2° . The yield was about 4% of the lipid P.

Fig. 6. Separation of cardiolipin and kephalin on silicic acid. Total P. Molar ratio P:N, fraction A, 1:0.03; fraction B, 1:0.94.

Table 6 shows the analyses of these preparations. The serological activity of O_5/b and $O_8/9S$ was tested by Dr A. E. Wilkinson and compared satisfactorily with that of a standard cardiolipin preparation, but extensive tests were not made. The calculated figures in Table 6 for alternative

Table 6. *Composition of cardiolipin preparations*

(a) Pangborn (1945); (b) Faure & Morelec-Coulon (1956). (c) $C_{120}H_{208}O_{24}P_3Na_3$: 5 linoleic, 1 oleic residue (Pangborn, 1947). (d) $C_{81}H_{136}O_{17}P_2Na_2$: 3 linoleic, 1 oleic residue.

	Present work			Other work		Calculated	
	O_5b	$O_7/16S$	$O_9/9S$	a	b	c	d
P (%)	4.1	3.8	4.1	4.18	4.1	4.23	4.17
Glycerol (%)	19.1	17.3	19.3	17.5	—	16.80	18.6
Fatty acids (%)	—	74	75	73.0	—	76.6	75.4
Molar ratio ($P=1.0$)							
Glycerol	1.57	1.53	1.56	1.40	1.5	1.34	1.5
Amino N	<0.03	0.08	<0.03	<0.03	<0.02	—	—
Inositol	Nil*	Nil*	Nil*	Nil*	Nil*	—	—

* Not detected.

structures of cardiolipin show the difficulty of determining the structure from the percentage composition.

Further work on the chromatographic purification is required. The method of fractionation on silicic acid is not entirely satisfactory. Cardiolipin is probably present as the acid in washed chloroform extracts and, even if the material is loaded on the column as the sodium salt, some material is eluted in a fast-running form free from cations; in this form, though the chemical composition is apparently unaltered, the solubility in ether and acetone and the serological activity progressively decrease (cf. Pangborn, 1944). This may be due to anhydride formation between neighbouring phosphoric acid groups, perhaps with some 'polymerization', for although a salt can be formed by immediate neutralization of the free acid it was impossible to make a salt from the 'polymerized' form. Moreover, although the cardiolipin band was clearly separated from the kephalin band, the cardiolipin still contained traces of nitrogenous and aldehyde derivatives, possibly because of occlusion.

The preparations of cardiolipin we obtained had a molar ratio of fatty acid ester to P of 2:1, but the molar ratio of glycerol to P was 3:2; this ratio of glycerol to P was also reported by Faure & Morelec-Coulon (1956). These results led us to suggest that cardiolipin contains three glycerol, two phosphoric acid and four fatty acid residues (structure V): in this structure, one glycerol residue is not fully esterified and the free hydroxyl has been assigned arbitrarily as a secondary alcoholic group on the middle glycerol. We tried to obtain evidence of structure in two ways (Macfarlane & Gray, 1957). First, an attempt was made

to oxidize the postulated hydroxyl group of structure (V) with potassium permanganate and examine the acid hydrolysis products for the oxidized fragment. Secondly, an attempt was made to determine the ratio of glycerol to glycerophosphate formed by saponification and acid hydrolysis of cardiolipin, which was expected to be 1:3 on Pangborn's (1947) structure and 1:2 on structure (V). These experiments were inconclusive and led us to examine the nature of the products formed from cardiolipin in different hydrolysis conditions.

Hydrolysis products of cardiolipin after alkaline or acid saponification

The experiments were based on the following argument. Removal of the fatty acids from structure (V), or from Pangborn's structure with four glycerol residues, gives a water-soluble polyglycerophosphate. On treatment with periodate the terminal glycerol residues will be oxidized to (VI) (or an analogous structure). On acid hydrolysis (VI) can give glycolaldehyde phosphate (rapidly converted into orthophosphate), glycerol, glycerolmonophosphate, glycolaldehyde or glycerol diphosphate, according to the manner of splitting of the P diester bonds (1, 2, 3 and 4). If the intact cardiolipin is hydrolysed in conditions in which the rate of splitting of the fatty acid ester bonds is similar to that of the P diester bonds, a variety of mono- and di-glycerides, as well as glycerol, glycerol monophosphate and glycerol diphosphate, may be formed.

Samples of cardiolipin $O_9/9S$ were saponified in different conditions, and the nature of the water-soluble phosphoric esters examined by determining

(a) the proportion of phosphomonoester, by hydrolysis with a phosphomonoesterase preparation, (b) the apparent proportion of P present as α -glycerophosphate, by oxidation with periodate at room temperature, and estimating the orthophosphate formed subsequently in 1 hr. in $N-H_2SO_4$ at 100° , (c) the apparent amount of P as total glycerol phosphate, by estimating orthophosphate after oxidation with periodate at 100° in $N-H_2SO_4$ for 1 hr. (Burmester, 1946). The conditions and results are shown in Table 7; all the P was recovered in the water-soluble fraction.

With mild alkaline conditions (Table 7A) the fatty acids are far more labile than the P diester linkages, and water-soluble polyglycerophosphate is formed. Only 40% of this appears as total glycerol phosphate by Burmaster's method. If the water-soluble polyester is first hydrolysed in $N-HCl$ and then oxidized (A, ii) 100% appears as phosphomonoester, but only 88% as apparent glycerol phosphate.

By contrast, when the cardiolipin was completely saponified in acid (Table 7E) all the P appeared as phosphomonoesters, but only 60% appeared as glycerol phosphate by periodate oxidation and hydrolysis. In acid the lability of the P diester bonds with glycerol is apparently of the same order as that of the fatty acid linkages, for when a sample of cardiolipin was heated 10 min. in $N-HCl$ at 100° only 60% of the P became water-soluble, and the fatty acid ester content of the ether-soluble portion was about 80% of the original. The presence of the substituent fatty acids appears to influence the manner of splitting of the P diester bands in acid in favour of formation of glycerol diphosphate, as only 60% of the P appeared as glycerol phosphate in E, compared with 88% in A ii. The formation of glycerol diphosphate was deduced from the results and has not been verified.

The kinetics of hydrolysis are clearly complex and may account in part for the inconclusive results previously reported. It is apparent that in analysis of material containing polyglycerol phosphatides, the estimation of 'glycerolphosphate P' by Burmaster's method may give misleading results.

Fatty acids present in cardiolipin

Two preparations of cardiolipin, $O_7/16S$, which still contained some nitrogenous impurity, and $O_6/9S$, which appeared to be free from kephalin and inositide, were saponified in $N-NaOH$ at 20° for 4 hr. under N_2 , and the fatty acids were extracted into ether, washed, dried, weighed and dissolved in methanol. Samples were taken for alkali isomerization and iodine values, and the remainder was converted into methyl esters which were examined chromatographically. The percentage composition of the mixed acids is shown in Table 8. The gas-liquid chromatograms did not distinguish satisfactorily between linoleic and linolenic acid but the sum of these acids agreed with the amounts calculated from the alkali-isomerization data; the amount of C_{20-22} acids were also in good agreement by the two methods, though the nature of the acids is not certain. The preparation $O_6/9S$ contained only 2.5% of saturated acids, while the less pure sample $O_7/16S$ contained about 8%; the percentage of saturated acids is perhaps as good a criterion of purity as any other, as the likely contaminants, kephalin and inositide, both contain about 50% of saturated acids. Palmitoleic and linolenic acids, as well as oleic acid, were present in the purest preparation $O_6/9S$, although, as found by Pangborn, the main acid present was linoleic. It is possible that cardiolipin, like the ester phosphatides, is a family rather than a single compound.

Table 7. *Nature of phosphorus esters formed from cardiolipin by hydrolysis in different conditions*

Conditions of saponification and hydrolysis	Nature of P esters (% of total)		
	Phosphomonoesters	Apparent glycerol monophosphate	
		α	$\alpha + \beta$
A. Methanolic $NaOH$, 0.2N, 15 min. at 37°			
(i) Water-soluble product	0	21	40
(ii) After hydrolysis of (i) in $N-HCl$, 10 min. at 100°	100	40	88
B. Ethanolic $N-NaOH$, 2 hr. at 38°	62	—	—
C. 0.5N- KOH in 95% ethanol, 2 hr. refluxing			
(a) Water-soluble product	100	47	93
(b) After hydrolysis of (a) in $N-HCl$, 10 min. at 100°	100	87	91
D. Aq. $N-NaOH$			
(i) 2 hr. at 38°	40	—	—
(ii) 1 hr. at 100°	89	—	—
(iii) 2 hr. at 100°	100	46	91
E. $N-HCl$ at 100° 2 hr.	100	42	60

Table 8. *Fatty acids of cardiolipin and glycerophosphoinositide*

(a) Gas-liquid chromatography by Dr A. T. James and Dr J. Webb. (b) Alkali-isomerization data.

Acid		Percentage of total fatty acids					
		Cardiolipin				Inositide	
		$O_7/16S$	$O_8/9S$				
(a)	(b)	(a)	(a)	(b)		(a)	(b)
C_{8-15}	—	1.12	Nil	—		Nil	—
$n C_{16}$	Palmitic	0.91	0.49	—		3.16	—
Branched unsaturated C_{17}	—	2.14	1.22	—		0.82	—
$n C_{17}$	—	—	—	—		0.73	—
$n C_{18}$	Stearic	2.67	0.79	—		45.95	—
Branched unsaturated C_{19}	—	0.96	—	—		—	—
Mono-unsaturated C_{16}	Palmitoleic	8.70	5.23	—		1.20	—
Mono-unsaturated C_{18}	Oleic	11.95	11.00	—		20.95	—
Diunsaturated C_{18}	Linoleic	59.50	80.00	70.8		12.26	7.63
Triunsaturated C_{18}	Linolenic	8.45				4.14	—
C_{20} (1)	Arachidonic	Tr.	0.74	1.24		10.78	10.94
(2)	C_{20-22} polyenoic	2.68	0.74	0.28		4.50	5.1
Total saturated acids		7.8	2.50	—		50.66	—
Total unsaturated acids		91.3	97.70	—		49.69	—

GLYCEROPHOSPHOINOSITIDE

The acidic phospholipid fractions from methanol extracts of heart muscle (similar to fraction A, Table 2) contained inositol equivalent to about 20 % of the P in the fraction. Attempts to crystallize inositide directly from this mixture in methanol, as described by Faure & Morelec-Coulon (1954), failed, but semicrystalline material apparently identical with the glycerophosphoinositide described by these authors was obtained from enriched fractions. On chromatographic fractionation of the acidic phospholipids of extract O_7 (p. 420) the rear fractions contained 50 % of inositide. These fractions were dissolved as the sodium salt (55 mg. of P) in 50 ml. ether, and methanol was added (about 8.0 ml.) until the mixture was turbid; after 6 days at -10° the semicrystalline deposit (161 mg.; P, 3.5; inositol, 16.8; glycerol, 12.0 %) was collected by centrifuging and washed with ether, in which it was insoluble. Another sample was obtained from the acidic phospholipid fraction of extract O_8 , after fractionation of the free acids with ethanol as described by Faure & Morelec-Coulon (1956); the ethanol-insoluble acids, containing kephalin and inositide and some cardiolipin, were redissolved in ether and the sodium salts (43 mg. of P) recovered by addition of sodium acetate and acetone; the ethereal solution (20 ml.) was treated with 2 vol. of methanol, and the mixture clarified by centrifuging: the supernatant, after several days at 2° , deposited semicrystalline material which was washed with ether, in which it was insoluble. Wt. 175 mg. (Found: P, 3.5; glycerol, 10.6; inositol, 19.9; fatty acids, 67 %; $[\alpha]_D^{25} + 7^\circ$ in chloroform (c, 1.88). Calc. for stearyl-linoleylglycerophosphoinositide; P, 3.5; glycerol,

10.4; inositol, 20.4; fatty acids, 64 %). The saturated and unsaturated fatty acids were separated as the lead salts insoluble and soluble in 95 % ethanol; the recovered fatty acids (55.5 % saturated, iodine no. 30; 44.5 % unsaturated, iodine no. 186) were examined by alkali isomerization; portions were also converted into methyl esters, which were analysed chromatographically. The results, calculated as a percentage of the total acids (Table 8), show that stearic acid accounts for nearly all the saturated acids, in agreement with Faure & Morelec-Coulon (1954); nearly one-third of the unsaturated acids were present as C_{20-22} polyenoic acids.

The yield of crystalline material from the acidic phospholipid fractions of O_7 and O_8 was only about 30 % of the total inositide in the fractions and no more was obtained on concentration of the mother liquors. It is possible that the crystalline inositide is a 'stearic' subfamily. No evidence that the inositol was present in a form different from a glyceromonophosphoinositide was obtained.

DISCUSSION

The method of adsorption chromatography on silicic acid developed by Lea *et al.* (1955) has been applied in the present work to the separation on Mallinkrodt silicic acid of phospholipids in extracts from ox heart containing high proportions of kephalin and choline plasmalogens. This method can be used without breakdown of plasmalogens if the phospholipid mixture is largely free from other lipid material. In the presence of the neutral fat breakdown of plasmalogens, apparently limited to breakdown of kephalin plasmalogen, can occur, though this is minimized by ageing the column.

The entire fractionation of a total lipid extract, e.g. a chloroform-methanol extract, on silicic acid by a combination of the methods of Borgström (1952) and Lea *et al.* (1955) carries the risk of breakdown, though this can be detected by the formation of free aldehyde. The application of the method for the quantitative separation of intact tissue phospholipids is therefore dependent on a quantitative separation of the phospholipids from neutral fat. For preparative work, preliminary defatting with acetone and extraction with methanol reduced the risk of breakdown of plasmalogens though the extracted phospholipid may not be wholly representative.

The phospholipids present in washed chloroform-methanol extracts of ox heart were quantitatively recovered from silicic acid columns with good separation into four main classes: (1) nitrogen-free polyglycerophosphatides 9%; (2) serine and ethanolamine phosphatides 43%, of which about 45% was plasmalogen; (3) choline phosphatides 42%, of which 55% was choline plasmalogen and (4) sphingomyelin 5%. Inositides were not present in the washed chloroform-methanol extracts but were present in methanol extracts of heart in amounts equivalent to 2% of the total lipid P. No effective separation of the plasmalogen component from the corresponding ester phosphatide takes place on silicic acid. Cardiolipin was separated from the kephalin phosphatides, but the method is not entirely satisfactory; though preparations of cardiolipin which were serologically active were obtained the substance runs partly or wholly as the free acid, in which form some change in nature can take place, and it may appear in a number of fast-running bands. An outstanding problem is the clean separation of the serine and ethanolamine phosphatides, the glyceroglycerolphosphatides and the glycerophosphoinositides from each other.

Though it has limitations, chromatography on silicic acid is very useful for the separate assay of kephalin and choline plasmalogen; and by subsequent degradation of the separated kephalin and choline phosphatides with acetic acid, and separation on silicic acid of the free aldehyde, the lyso-phosphatide and the intact ester phosphatide, the fatty components of the plasmalogens and the ester phosphatides can be recovered separately.

In attempts to separate the intact plasmalogen from the corresponding ester phosphatide, the use of partition chromatography with secondary cellulose acetate as support was examined. With a cyclohexane-methanol solvent system considerable enrichment was achieved, fractions containing up to 90% of plasmalogen being obtained. However, the iodine value indicated that members of plasmalogen and lecithin families had been separated according to the degree of unsaturation, and

possibly also of chain length, of the constituent fatty acids. It appears that it would be very difficult to obtain pure plasmalogen which in its fatty components was representative of the whole content.

Simple phosphatidic acids, such as might be intermediates in the synthesis or metabolism of lecithin and kephalin, and lysolecithin were not detected in the ox heart extracts examined, and the presence of appreciable quantities of lysokephalin was traced to the breakdown of kephalin plasmalogen on the column. The plasmalogens appear to be stable in the excised tissue, which was not extracted with solvents till 2-3 hr. after death. The significance of the large proportions of choline and ethanolamine plasmalogens in heart is unknown. The differences in the fatty acids of the plasmalogens and the ester phosphatides found by Klenk & Krickau (1957) and Gray (1958*a*) and the characteristic fatty acid pattern of cardiolipin and glycerophosphoinositide focus attention also on the selectivity towards the fatty components involved in the biosynthesis of these phospholipids.

SUMMARY

1. Phospholipids equivalent to about 560 mg. of phosphorus/kg. were extracted from ox heart with chloroform-methanol mixtures and the washed lipid was fractionated on Mallinkrodt silicic acid columns.

2. The phospholipids, in mixtures which contained about 45% of plasmalogens but no inositides, were recovered quantitatively with good separation into cardiolipin, kephalin, lysokephalin, cholinephosphatides and sphingomyelin. The kephalin and choline phosphatide fractions contained about 45 and 55% respectively of plasmalogens which were not separated to any useful extent from the corresponding ester phosphatide.

3. In the presence of the neutral fat fraction, kephalin plasmalogen is liable to decompose on silicic acid columns, with formation of free aldehyde and lysokephalin. Apart from this decomposition, lysophosphatides were not detected.

4. On treatment of acetone-defatted heart with methanol, 95% of the choline phosphatides, together with cardiolipin and inositide, but only 70% of the kephalin was extracted.

5. A method for partition chromatography of phospholipids with organic solvents and secondary cellulose acetate as support is described. With cyclohexane-methanol systems, fractionation of lecithin-choline plasmalogen mixtures containing 50% of plasmalogen yielded 20% of the total lipid in fractions containing 75-85% of plasmalogen. Evidence that the separation is influenced by the nature of the fatty acids was obtained.

6. Data on the composition of cardiolipin preparations and on the mode of hydrolysis of cardiolipin in acid and alkaline conditions are recorded.

7. Glycerophosphoinositide equivalent to about 30 % of the inositide present was obtained as crystals from inositide-rich fractions. This inositide contained saturated and unsaturated acids in equimolar proportions: 46 % of the fatty acids was present as stearic acid and 15 % as C₂₀₋₂₂ highly unsaturated acids.

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The Structure of the Plasmalogens of Ox Heart

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Although the natural plasmalogens have not yet been obtained as pure substances, the work of Klenk & Debuch (1954, 1955) and Debuch (1956) has shown conclusively that these plasmalogens contain a fatty acid as well as a fatty aldehyde residue. On treatment of fractions containing both the classical ester phosphatide and the plasmalogen with dilute acid to remove the aldehyde, lysolecithin was obtained from the choline-phosphatide

fraction of ox-heart muscle (Klenk & Debuch, 1955) and lysokephalin from the fraction of brain (Debuch, 1956). Klenk & Debuch (1954) had earlier shown that after catalytic hydrogenation of the mixed kephalin fraction the aldehydic properties disappeared, and on alkaline or acid hydrolysis 60 % of the phosphorus was present as chimyl or batyl alcohol phosphoric ester, i.e. as glyceryl ether derivatives. They suggested (I) or (II) as