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Phosphatides of Normal Human Serum

Part I. Fractionation of the Phosphatides *,**

O. RENKONEN

Department of Serology and Bacteriology University of Helsinki, Finland

Phosphatides of pooled, normal human serum were fractionated with chromatographic and hydrolytic methods. The following phosphatides were isolated in nearly pure state: phosphatidylcholines, lysophosphatidylcholines, phosphatidylethanolamines, lysophosphatidylethanolamines, phosphatidylinositols, lysophosphatidylinositols, acylated α-alkoxy-glycerophosphorylcholines, and finally, sphingomyelins. In addition concentrates of an unknown acidic glycerophosphatide as well as of native choline and ethanolamine plasmalogens and of acylated alkoxy-glycerophosphorylethanolamines were isolated.

Phosphatidylcholines, phosphatidylethanolamines, sphingomyelins, and lysophosphatidylcholines have been isolated from normal human serum in several laboratories.[3–9] Many other phosphatides have also been observed in serum,[3,10–13] but they have not been isolated for thorough chemical characterization. This report describes a fractionation experiment which led to preparative isolation and characterization of eight different types of serum phosphatides.

RESULTS

A lipid extract was prepared from 2300 ml serum of 18 healthy, fasting donors.*** It contained 17.7 g lipids, and preliminary analyses of its phosphatide components revealed 230 mg total phosphorus and 28 mg bound inositol.

* A summary of this report was presented at the 11th Meeting of Scandinavian Chemists, Turku, Finland, August 1962. Two preliminary communications concerning a part of the present investigation have been published elsewhere.[1,2]

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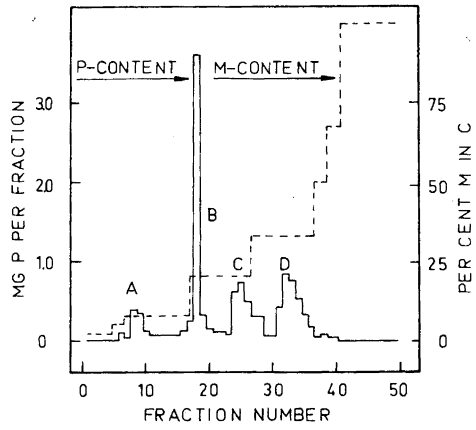

Fig. 1. Chromatography of the "cephalins" on 70 g silicic acid (26 × 270 mm). (Run No. 4) Fraction volume: 100 ml. Load: 12.2 mg P, 17.1 mg inositol. Recovery: 12.1 mg P, 17.9 mg inositol.

Chromatographic fractionation. The serum extract was first fractionated with silicic acid chromatography [3] into neutral lipids, "cephalins",* lecithins, sphingomyelins and lysolecithins, whereafter a closer study of the "cephalins" was undertaken.

When the "cephalins" were rechromatographed on silicic acid an elution pattern of four distinct phosphorus peaks was obtained (Fig. 1). Paper chromatography showed that Peak A contained acidic phosphatides, unknown at this stage of the work, whereas Peak B contained phosphatidylethanolamines, Peak C their lyso derivatives, and Peak D lecithins. However, inositol analysis revealed that the inositides were rather evenly distributed in all these peaks. Therefore the fractions in Fig. 1 were recombined to form only two major preparations for further fractionation.

The combined fractions eluted before Peak D in Fig. 1 gave the elution pattern shown in Fig. 2 (Run No. 5) when chromatographed on alumina.[14,15]. Here the Peak E fractions contained a partially resolved mixture of several lipids including phosphatidylethanolamines, their lyso derivatives, an unknown acidic phosphatide ("Phosphatide X") and a group of unidentified nonphosphatides ("Z-Compounds"). The Peak F fractions, on the other hand, contained nearly pure phosphatidylinositols.

Also the Peak D material in Fig. 1 was resolved on alumina into two peaks, which are shown in Fig. 2 (Run No. 6). Here the Peak G material was pure lecithin, whereas the Peak H fractions contained substantially pure inositol lipids, which in this case proved to be lysophosphatidylinositols.

Now that the inositol lipids had been removed, the rest of the "cephalins", *i.e.* the Peak E material in Fig. 2, could be resolved on silicic acid into a concentrate of "Phosphatide X", a preparation of impure phosphatidylethanolamines, and a fairly pure sample of lysophosphatidylethanolamines.

An attempt was finally made to purify the phosphatidylethanolamines on DEAE-cellulose.[16] However, the contaminating "Z-Compounds" were only

* The terms and abbreviations used are explained in the experimental part.

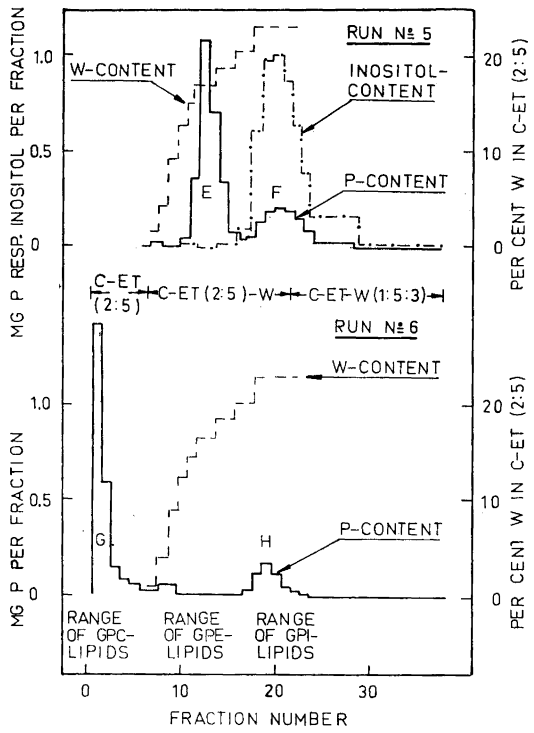

Fig. 2. Run No. 5: Chromatography of Fr. 6—28/4 on 10 g aluminum oxide (16 × 60 mm). Fraction volume: 50 ml. Load: 3.83 mg P, 6.19 mg inositol. Recovery: 3.71 mg P, 5.54 mg inositol.
Run No. 6: Chromatography of Fr. 30—41/4 on 10 g aluminum oxide (16 × 60 mm). Fraction volume: 50 ml. Load: 3.30 mg P, 3.96 mg inositol. Recovery: 3.22 mg P, 3.14 mg inositol.

slightly enriched into the rear fractions of the phosphatidylethanolamine peak. Even repeated chromatograms failed to yield quite pure phosphatidylethanolamines, but they were nevertheless pure enough for characterization and identification, for the "Z-Compounds" appeared to be nonphosphatides free of acid and alkali labile groups.*

Hydrolytic fractionation. Analysis of the serum lecithins revealed that they were a mixture of three different types of closely related phosphatides (Fig. 3). Besides the common phosphatidylcholines (I) this preparation also contained about 1 % native choline plasmalogens (II), and about 2 % acylated α-alkoxy-glycerophosphorylcholines (III).

This mixture could be partially fractionated with selective "chemical" hydrolyses.[2,17-20] When it was subjected to mild alkaline treatment the phos-

* The slower migrating "Z-Compounds" were isolated with preparative thin layer chromatography. They were free of phosphorus. All "Z-Compounds" appeared to be unchanged after mild alkaline and acid hydrolyses.

phatidylcholines were deacylated more rapidly than the other two lecithin types, and after a while the hydrolysate contained the five phosphorus compounds (II—VI) shown in Fig. 3. Partition [1] of the hydrolysate then separated

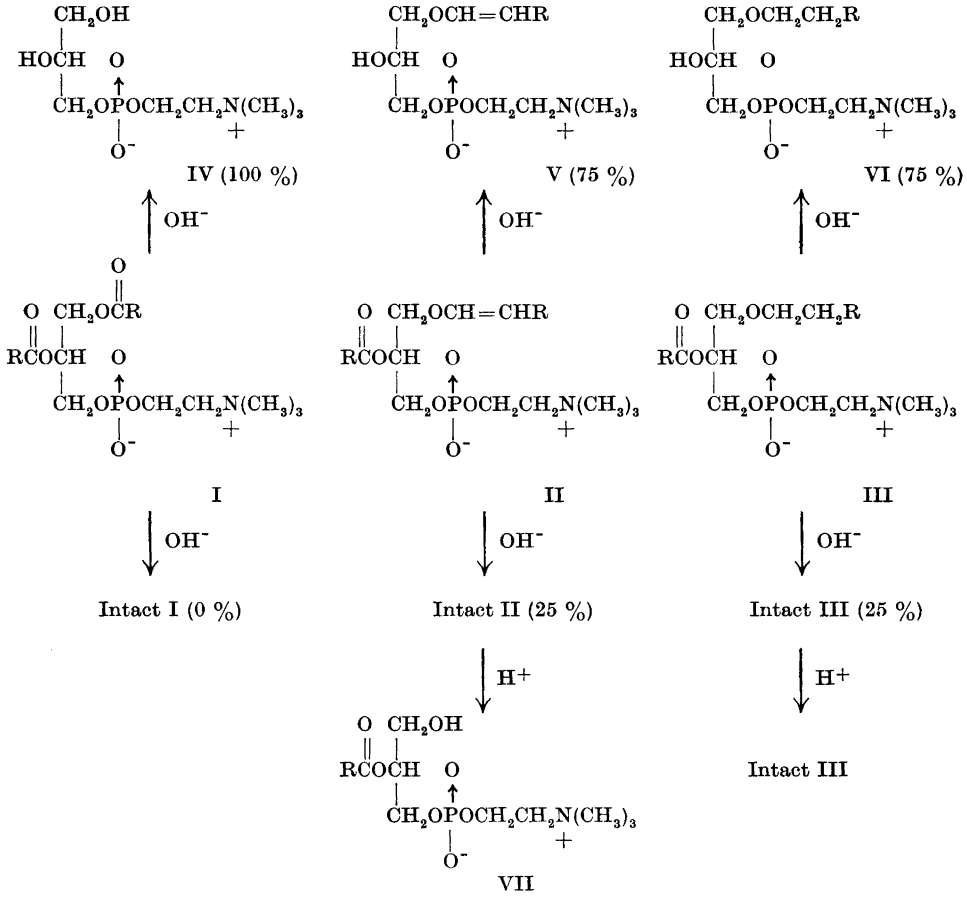

Fig. 3. Reaction scheme showing the hydrolytic fractionation of serum lecithins.

the water soluble glycerophosphorylcholines (IV) from the lipid soluble phosphatides (II, III. V and VI). These were then separated with silicic acid chromatography into two fractions, the first of which contained the "two chain" derivatives (II and III), and the second the corresponding deacylated lipids (V and VI). The mixture of the native plasmalogens (II) and alkoxy phosphatides (III) was thus obtained in about 25 % yield despite their low concentration in the starting material. The acylated alkoxy glycerophosphorylcholines (III) were finally isolated in pure form by hydrolyzing the contaminating plasmalogens (II) by mild acid treatment into free aldehydes and lysophos-

Table 1. Hydrolytic fractionation of serum lecithins.

Fractionation steps [a]	µg P [b]	TLCh.[c]	Molar ratios: Acyl ester / P	Alkali lab. P / P	Acid lab. P / P	Apparent glycerol / P
Original serum lecithin						
– Prep. 1: EE–GPC+AE–GPC+VE–GPC	15400	A-2	2.10	0.95		1.02
Alkaline hydrolysis of Prep. 1						
– Prep. 2: AE–GPC+VE–GPC	192	A-2	1.00		0.02	0.31 [d]
– Prep. 3: A–GPC+V–GPC	464	A-1	0.02	0.04	0.30	0.47
Acid hydrolysis of Prep. 2 [d]						
– Prep. 4: AE–GPC	147	A-2	1.07	0.05		0.18
– Prep. 5: E–GPC	32	A-1	1.15			

a) The symbols for the different lipids are explained in the experimental part.
b) The yields indicated correspond to the whole starting material.
c) A-2 is the spot common to all "two-chain" derivatives of GPC, whereas A-1 is the spot of the corresponding "mono-chain" derivatives.
d) The glycerol analysis as well as the mild acid hydrolysis suggest that the ratio of AE–GPC to VE–GPC in this preparation is about 3:1. This has been later confirmed with other similar preparations by using also the spectrophotometric iodine method [40] and the phosphorus partition method. [19]

phatidylcholines (VII); the intact alkoxy phosphatides were then easily isolated from the hydrolysate with silicic acid chromatography.

The actual results of the fractionation of serum lecithins are summarized in Table 1. The identification of the acylated alkoxy-glycerophosphorylcholines is based on their chromatographic similarity to pure phosphatidylcholines and native choline plasmalogens,[19] their equimolar content of carboxylic ester and phosphorus, and their behaviour under prolonged alkaline hydrolysis and strong acid hydrolysis; the prolonged alkaline treatment gave good yield of deacylated alkoxy-glycerophosphorylcholines, whereas the strong acid hydrolysis gave little free glycerol but released a mixture of simple glyceryl ethers.

The location of the ether groups was determined by acetolysis [21] of the deacylated phosphatides, alkaline hydrolysis of the acetylated diols formed, and subsequent periodate oxidation of the free diols. The formation of one mole of formaldehyde proved that the alkoxy groups very likely were in the α-position.

The native choline plasmalogens (II) were not yet obtained in pure form, free of the alkoxy phosphatides, but their presence in serum became firmly established during the isolation of the alkoxy phosphatides.

Also the serum phosphatidylethanolamines proved to be accompanied by the corresponding native plasmalogens and alkoxy phosphatides, and this mixture could be fractionated by mild alkaline treatment quite as the lecithin

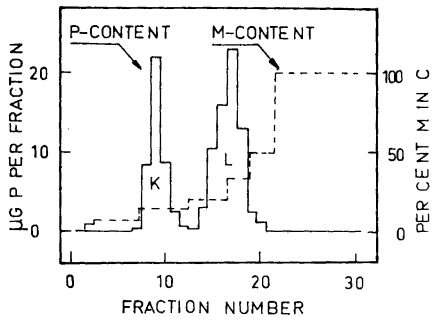

Fig. 4. Chromatography of the alkali stable phosphatides from the phosphatidylethanolamine hydrolysate on 2.0 g silicic acid (10 × 47 mm). Fraction volume: 5 ml. Load: 119 μg P. Recovery: 112 μg P.

preparation. Here too the diacyl phosphatides were deacylated more rapidly than the other "two chain" derivatives of glycerophosphorylethanolamine, and when the remaining phosphatides were extracted and subjected to silicic acid chromatography the elution pattern shown in Fig. 4 was obtained. The Peak K fractions (Prep. 7 in Table 2) contained a mixture of about equal parts of native ethanolamine plasmalogens and corresponding alkoxy phosphatides (native "Cephalin B" [20]). These fractions were, however, contaminated by the alkali stable "Z-Compounds". The Peak L fractions (Prep. 8 in Table 2), on the other hand, contained the deacylated forms of the two phosphatides in fairly pure state.

The presence of both plasmalogens and alkoxy phosphatides in the alkali stable fractions was established as follows. The Peak K material, was subjected

Table 2. Hydrolytic fractionation of serum GPE-lipids.

Fractionation steps [a]	μg P [b]	TLCh.[c]	Molar ratios			
			Acyl ester P	Alkali lab. P P	Acid lab. P P	Apparent glycerol P
Original serum GPE-lipids						
— Prep. 6: EE—GPE+AE—GPE+VE—GPE+Z	724	B-2 + Z	1.72	0.80	0.01	0.91
Alkaline hydrolysis of Prep. 6						
— Prep. 7: AE—GPE+VE—GPE+Z	43	B-2 + Z	0.97		0.05	
— Prep. 8: A—GPE+V—GPE	72	B-1	0.03	0.23	0.48	0.26
Acid hydrolysis of Prep. 7						
— Prep. 9: AE—GPE+Z	20	B-2 + Z		0.23		
— Prep. 10: E—GPE	17	B-1		0.98	0.08	

a) The symbols for the different lipids are explained in the experimental part.
b) The yields indicated correspond to the whole starting material.
c) B-2 is the spot common to all "two-chain" derivatives of GPE, B-1 is the spot of the corresponding "monochain" derivatives, and Z corresponds to the "Z-Compounds".

Table 3. Characterization of the serum phosphatides.

Lipid [a]	Approx. amount in 1000 ml serum (mg P)	TLCh. & PCh.[b]	Molar ratios					
			Acyl ester P	Apparent glycerol P	Alkali lab. P P	Acid & alkali lab. P P	Acid lab. P P	Inositol P
EE–GPC	62	A-2	2.00	1.06	0.95	0.96		
AE–GPC	1.3	A-2	1.07	0.18	0.05	0.04		
E–GPC	10	A-1	1.02	1.09	0.97		0.08	
EE–GPE	1.5	B-2 + Z	1.72	0.91	0.80	0.89	0.01	
AE–GPE	0.2	B-2 + Z				0.23		
E–GPE	1.2	B-1	1.11	1.09	0.98		0.08	
EE–GPI	1.4	C-2	1.82	1.03				1.03
E–GPI	0.7	C-1	1.06	1.12				0.94
SPH	17	SPH	0.08	0.05	0.00	0.00		
X	Trace	X [c]	2.56	1.15				

a) The symbols for the different lipids are explained in the experimental part.

b) A-2 is the spot common to all "two chain" derivatives of GPC, B-2 and C-2 are the corresponding spots of GPE and GPI lipids. A-1, B-1 and C-1 denote the spots of the "mono chain" derivatives.

c) "Phosphatide X" stained like acidic phosphatides on PCh.; it was not identical with phosphatidic acid, phosphatidylserine, phosphatidylglycerol or cardiolipin.

to mild acid hydrolysis (Table 2) and the lipids of the hydrolysate were extracted and fractionated by silicic acid chromatography. The first of the two phosphatide peaks thus obtained contained native "Cephalin B", which had remained intact but which still was grossly contaminated with the stable "Z-Compounds". The second phosphatide peak contained pure lysophosphatidylethanolamines formed from the native ethanolamine plasmalogens. The impure native "Cephalin B" (Prep. 9 in Table 2) was finally subjected to prolonged mild alkaline hydrolysis, which caused only insignificant liberation of water soluble phosphorus, but changed the lipid so that on thin layer chromatography the original spot of the native "Cephalin B" disappeared and the spot of the "mono chain" derivatives of glycerophosphorylethanolamine appeared. This proved that the native "Cephalin B" of serum really contained the expected alkali labile carboxylic ester grouping in addition to its acid and alkali resistent fatty chain linkage.

Further evidence for the presence of "Cephalin B" in serum was obtained by study of the Peak L material in Fig. 4 (Prep. 8 in Table 2). Mild acid hydrolysis converted about half of the phosphorus of this material (the lysoplasmalogens) into water soluble phosphates, whereas the other half remained lipid soluble. This stable fraction showed only the spot of the "mono chain" derivatives of glycerophosphorylethanolamine on thin layer chromatography, and like all alkoxy phosphatides it liberated very little free glycerol on strong acid hydrolysis, instead of which simple glyceryl ethers were detected in the hydrolysate.[1]

Characterization of the phosphatides isolated. Most of the phosphatides discussed were isolated in fairly pure form during the fractionation described above. Their characterization could thus be carried out after pooling the respective chromatographic fractions. Only the sphingomyelins and the phosphatidylinositols required further purification, which is described in the experimental part. The principal results of the characterization of the phosphatides isolated are given in Table 3. The analysis of the fatty chains of these lipids, will be described elsewhere.*

EXPERIMENTAL

The following abbreviations are used: TLCh. = thin layer chromatography, PCh. = paper chromatography, H = heptane, C = chloroform, M = methanol, Et = ethanol, W = water, AcOH = acetic acid, GPC = glycerophosphorylcholine, GPE = glycerophosphorylethanolamine, GPI = glycerophosphorylinositol, EE–GPC = phosphatidylcholine,** VE–GPC = native choline plasmalogen,* AE–GPC = acylated alkoxyglycerophosphorylcholine,* E–GPC = lysophosphatidylcholine, V–GPC = choline lysoplasmalogen, A–GPC = alkoxy-glycerophosphorylcholine, SPH = sphingomyelin, lecithin = mixture of EE–GPC, VE–GPC and AE–GPC, "cephalins" = choline free glycerophosphatides; they are symbolized in the same way as the GPC lipids.

The composition of solvent mixtures is specified in volume ratios.

Analytical methods. Phosphorus,[22] stable and labile phosphorus after mild acid, mild alkaline, and succesive mild acid and alkaline hydrolysis,[19] as well as carboxylic esters,[23] glycerol,[24] and inositol [25] were determined by the procedures indicated.

Chromatographic methods. Column chromatography of phosphatides on silicic acid,[26] on alkali free alumina,[27] and on DEAE-cellulose [16] was carried out at 20° with stepwise gradient elution with C–M or C–Et–W mixtures.

Silicic acid (Mallincrodt) was pretreated by removing the smallest particles as described by Hirsch and Ahrens,[4] after which the preparation was activated at 110° for 16 h. The activity of the adsorbent used was characterized by shaking 5 g samples with 30 ml C–Et (995:5) containing 50 mg cholesterol. Under these conditions 67 % of the cholesterol was adsorbed. The activity of the pretreated [27] alumina was measured similarly except that H–C (4:6) was used as solvent. The preparation adsorbed 22 mg cholesterol. Woelm's neutral aluminium oxide, grade III, had very similar activity; it adsorbed 23.5 mg cholesterol under these conditions. DEAE-cellulose was pretreated as described by Rouser *et al.*[16]

The isolation of representative phosphatide samples required special attention, because silicic acid is known to cause some fractionation even inside certain phosphatide classes.[9,28] The preservation of the original fatty chain "spectrum" of the different phosphatides through the whole chromatographic isolation procedure was ascertained by cutting the chromatograms in fractions of equal volume, and by recombining them again on equal volume basis for rechromatography or characterization.

PCh.[29] and TLCh.[30] of phosphatides were carried out as indicated; staining with Rhodamine 6 G or by charring with 50 % H_2SO_4. For TLCh. of inositides, however, an acidic system,[31] Kieselgel G/propionic acid-C-propanol-W (15:15:21:7) was used. It separated EE–GPI and E–GPI clearly from each other and also from the stationary polyphosphoinositides of ox-brain.

Extraction of the lipids. Serum was extracted by scaling up our laboratory's modification [25] of the analytical procedure of Bragdon.[32] This modification gives essentially the same yield of phosphatides from normal human serum as the method of Bragdon despite the remarkably reduced ratio of solvent to serum.

Silicic acid chromatography of the total lipids. Preliminary fractionation of the serum lipids was carried out with silicic acid chromatography (Run No. 1, Table 4). Fr. 3–4/1 were combined for rechromatography on silicic acid. Fr. 5–7/1 were later combined

* E. L. Hirvisalo and O. Renkonen; manuscript under preparation.

** E denotes a carboxylic *ester* group, V a *vinyl ether* group, and A an *alkoxy* group.

Table 4. Chromatography of serum lipids on 250 g silicic acid (50 × 270 mm). (Run No. 1). Fraction volume: 500 ml. Load: 15.9 g lipids, 207 mg P, 25 mg inositol. Recovery: 202 mg P, 24 mg inositol.

Fr. No.	Eluent	Principal lipids eluted	mg P	Molar ratios				
				Acyl ester / P	Alkali lab. P / P	Acid & alkali lab. P / P	Apparent glycerol / P	Inositol / P
1	C	Neutral	0.08					
2	C—M (9:1)	lipids	0.06					
3—4	C—M (9:1)	Mixture	1.60					0.68
5—7	C—M (3:1)	"Cephalins"	11.4	1.77			1.01	0.17
8—10	C—M (3:1)	Lecithins	82.2	2.09	0.953	0.964	1.02	0.011
11—12	C—M (37:13)	»	28.0	1.96	0.961	0.975	1.01	0.003
13	C—M (36:14)	»	9.90	2.02	0.950	0.975	1.03	0.002
14	C—M (35:15)	»	7.20	1.80			0.95	
15	C—M (34:16)	Mixture	5.00	1.49		0.77	0.80	
16—17	C—M (33:17)	SPH	13.3	0.25		0.07	0.14	
18—22	C—M (1:1)	SPH	22.1	0.07		0.03	0.06	
23	C—M (1:1)	Mixture	1.24	0.70			0.76	
24—27	C—M (1:1)	E—GPC	14.9	1.01	0.974	0.981	1.04	
28—30	M	»	5.16	1.00			1.02	
31—33	M		0.17					

with other "cephalin" fractions. Fr. 8—10/1 were combined for refractionation on silicic acid. Fr. 11—14/1 were later combined with other lecithin fractions. Fr. 16—22/1 served for final purification and isolation of SPH. Fr. 24—30/1, which contained pure E—GPC, were pooled for characterization (Table 3).

Rechromatography (Run No. 2) of Fr. 3—4/1 (1637 mg lipids, 1580 μg P, 6270 μg inositol) on 60 g silicic acid gave 1474 mg nonphosphatides (Fr. 1/2; eluted with C containing 2—5 % M) and a phosphatide preparation (Fr. 2/2; 1520 μg P, 6000 μg inositol; eluted with C containing 5—50 % M). Fr. 2/2 was combined with Fr. 5—7/1.

Rechromatography (Run No. 3) of Fr. 8—10/1 (74 mg P, 4500 μg inositol) on 200 g silicic acid gave a small preparation rich in inositol lipids (Fr. 1/3; 780 μg P, 2100 μg inositol) which was eluted just in front of the lecithin peak (Fr. 2/3; 73 mg P, 1800 μg inositol). Fr. 1/3 was combined with Fr. 5—7/1, and Fr. 2/3 was added to Fr. 11—14/1. A sample of Fr. 2/3 served also for the isolation of VE—GPC and AE—GPC.

Silicic acid chromatography of the "cephalins". A representative sample of the serum "cephalins" was obtained by combining Fr. 5—7/1, 2/2, and 1/3. This sample contained 88.5 % of the obtainable "cephalins" of each of the Fr. 3—10/1. Rechromatography (Run No. 4) of this sample on silicic acid gave the elution pattern shown in Fig. 1. The molar ratio of inositol to P in Peak A was 0.60, in Peak B 0.18, in Peak C 0.08, and in Peak D 0.20.

Separation of the inositol lipids. Fr. 6—28/4, *i.e.* Peaks A, B, and C of Fig. 1, were recombined and fractionated with two identical alumina chromatograms, one of which is presented in Fig. 2. The total material fractionated (7.65 mg P) yielded thus 5.19 mg P Peak E and 2.09 mg P Peak F phosphatides. The Peak E material was still a complicated mixture of different lipid types, but the Peak F fractions contained fairly pure EE—GPI.

Fr. 30—41/4, *i.e.* Peak D material in Fig. 1, were recombined and chromatographed on alumina (Run No. 6; Fig. 2). Fr. 1—7/6 contained pure lecithins (2.39 mg P). They were combined with Fr. 11—14/1 and Fr. 2/3, after which the total lecithins could be characterized (Table 3). Fr. 18—21/6 contained substantially pure E—GPI (459 μg P) which were directly characterized (Table 3).

Fractionation of the inositol free "cephalins". The fractionation of Peak E lipids (4.76 mg P) on silicic acid was carried out essentially in the same way as the original Run No. 4 of the total "cephalins". The principal fractions thus obtained were Phosphatide X (49 μg P), eluted with C—M (12:1), EE—GPE (2.43 mg P), eluted with C—M (4:1), and E—GPE (1.78 mg P), eluted with C containing 20—33 % M. The other preparations were characterized (Table 3) but EE—GPE which was still contaminated with the "Z-Compounds" and possibly with traces of "Phospatide X", was subjected to further purification.

Final purification of phosphatidylethanolamines. The contaminated EE—GPE (1448 μg P) was chromatographed on 21 ml DEAE-cellulose (acetate form)-Kieselgur (1:1) (v/v). This separated a small amount (51 μg P) of an acidic lipid which was eluted first with AcOH and resembled "Phosphatide X" on PCh. The EE—GPE (1225 μg P) was eluted already with C containing 11—30 % M. As it was still contaminated with the "Z-Compounds" it was rechromatographed three times on DEAE-cellulose. These chromatograms gave the EE—GPE preparation (Fr. 1/12; 970 μg P), which was characterized (Table 3). Even this preparation was, however, contaminated with small amounts of the "Z-Compounds", and in addition it contained also VE—GPE and AE—GPE.

Final purification of sphingomyelins. A sample of Fr. 16—22/1 (7.35 mg P) was dissolved in 150 ml of C—M (1:1) and treated with 25 ml 0.35 N NaOH at 20° for 45 min, when 74 ml W, 1 ml M and 125 ml C were added, whereafter the mixture was shaken and centrifuged. The lower liquid layer was separated, washed twice with 30 ml of fresh upper phase, dried with Na_2SO_4, evaporated to dryness under nitrogen and dissolved in C. The stable phosphatides (6.76 mg P) were then subjected to silicic acid chromatography for the elimination of traces of methylesters, deacylated plasmalogens and alkoxy-phosphatides. Pure SPH (6.32 mg P) was thus obtained for characterization (Table 3).

Final purification of phosphatidylinositols. A sample (389 μg P) of Fr. 17—28/5 was chromatographed on silicic acid (Run No. 13; Fig. 5). Fr. 8—26/13 which contained pure EE—GPI (273 μg P) were characterized (Table 3). Fr. 47—50/13 contained pure E—GPI (48 μg P); upon analysis they showed equimolar amounts of inositol, carboxylic esters, glycerol and phosphorus.

Preparation of lysophosphatidylinositol used as chromatographic marker. A chromatographic marker for E—GPI on TLCh was obtained by subjecting authentic EE—GPI *

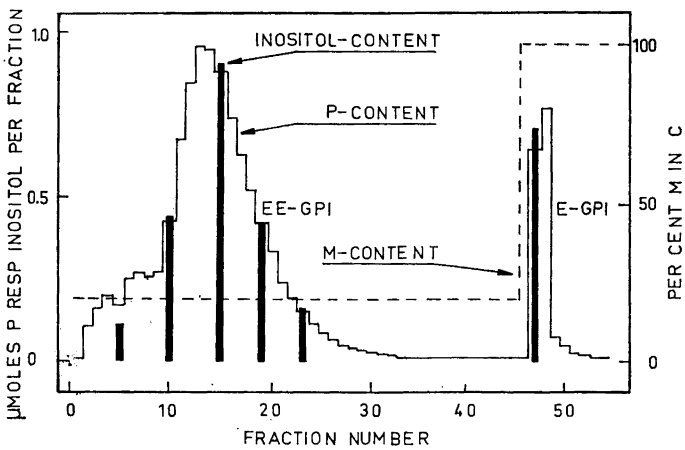

Fig. 5. Chromatography of Fr. 17—28/5 on 4.0 g silicic acid (13 × 70 mm). (Run No. 13.) Fraction volume: 5 ml. Load: 389 μg P. Recovery: 372 μg P.

* The sample was kindly donated by Dr. M. Faure, Paris.

to partial alkaline deacylation [33] in 0.05 N NaOH in moist C—M (3:4). The reaction was stopped after 2—5 min by transferring aliquots of the solution to starting points of TLCh-plates which contained a little propionic acid. The reaction mixture showed two spots on the developed plates; unchanged EE—GPI and a slower migrating component, considered to be E—GPI. Similarly, EE—GPC gave a mixture of EE—GPC and E—GPC under these conditions.

Partial alkaline hydrolysis of lecithin. A sample (15.4 mg P) of Fr. 2/3 was treated with NaOH in the same way as the SPH above. The alkali stable phosphatides obtained (688 μg P) were chromatographed on 2.0 g silicic acid (Run No. 14). Fr. 6—8/14 (164 μg P), eluted with C—M (1:1), contained AE—GPC and VE—GPC (Table 1). Fr. 9—10/14 (37 μg P) were mixtures of AE—GPC, VE—GPC, A—GPC, and E—GPC. Fr. 11—18/14 (440 μg P), eluted with C—M mixtures containing 67—100 % M, consisted of A—GPC and V—GPC (Table 1).

Mild acid hydrolysis of the mixture of AE—GPC and VE—GPC. A sample of Fr. 6—8/14 (106 μg P) in 1.50 ml C was treated with 1.50 ml of 0.1 N HCl in 99 % M for 60 min at 20°, after which 2.50 ml C, 0.515 ml M and 1.485 ml W were added. Equilibration of the mixture and separation of the two layers gave 1.82 μg water soluble P and 97 μg lipid soluble P. The stable phosphatides (93 μg P) were then chromatographed on silicic acid (Run No. 15). Fr. 6—9/15 (72 μg P), eluted with C—M (1:1), contained pure AE—GPC (Table 1). Fr. 10—12/15 (3 μg P) were mixtures of AE—GPC and E—GPC. Fr. 13—17/15 (15 μg P), eluted with C—M (1:2) and M, contained pure E—GPC (Table 1).

Strong acid hydrolysis of AE—GPC. A sample of Fr. 6—9/15 (0.41 μmoles P) was subjected to strong acid hydrolysis under the conditions used for the analytical determination of glycerol. When the aqueous phase of the extracted hydrolysate was oxidized with periodate only 0.07 μmoles of apparent glycerol were detected. But when the C extract of the hydrolysate was subjected to TLCh in system diethyl ether-AcOH (100:2) Kieselgel G, a strong spot with the same mobility as batyl alcohol (R_F = 0.62) was observed.

Mild alkaline hydrolysis of AE—GPC. A sample (30 μg P) of Fr. 6—9/15 was treated with NaOH in the same way as the SPH above, but all solvent volumes were scaled down 50-fold, and the reaction was continued for 90 min. A sample (25 μg P) of the lipid soluble fraction (27 μg P) of the hydrolysate was chromatographed on 2.0 g silicic acid (Run No. 16). Fr. 7—9/16 (3 μg P), eluted with C—M (1:1), contained unchanged AE—GPC, whereas Fr. 13—18/16 (20 μg P), eluted with C containing 75—100 % M, contained pure A—GPC, which showed only the spot common to all "monochain" derivatives of GPC on TLCh.

Acetolysis of A—GPC. Additional A—GPC was prepared from Fr. 2/3 by combined acid and alkaline hydrolysis as described elsewhere.[2] A sample (4.5 μmoles) of this preparation [2] was subjected to acetolysis under the conditions of Carter *et al.*[21] The reaction mixture was taken to dryness in reduced pressure under nitrogen, partitioned between 20 ml C and 5 ml W, and the C layer was washed with W, dried with Na_2SO_4, and passed through 0.5 g alumina in 50 ml C—M (1:1), whereafter the filtrate was chromatographed on 2 g silicic acid (Run No. 17). Only Fr. 5/17, eluted with C—M (9:1), contained acyl esters (7.1 μmoles). This fraction was identical with di-O-acetyl-batylalcohol on TLCh. (System: Petroleum ether-diethyl ether (1:1)/Kieselgel G; R_F = 0.72).

Alkaline hydrolysis of di-O-acetyl-glyceryl ethers. A sample (2.82 μmoles carboxylic ester) of Fr. 5/17 was treated with NaOH in the same way as the SPH above, but all solvent volumes were scaled down 50-fold. The C-layer of the partition contained the free glyceryl ethers. We believe that the procedure resulted in quantitative saponification and extraction, for our model compound, di-*O*-acetyl-batylalcohol, was completely hydrolyzed and quantitatively extracted under these conditions. Therefore we assume that the C-layer contained 1.41 μmoles of free glyceryl ethers.

Periodate oxidation of the free glyceryl ethers. A sample (0.94 μmoles) of the free glyceryl ethers derived from A—GPC was dissolved in 2.0 ml AcOH—W (9:1) and treated with 0.1 ml 10 N H_2SO_4 and 0.50 ml 0.1 M $NaIO_4$ for 5 min at 20° in the dark. The reaction was stopped by adding 0.50 ml 10 % $NaHSO_3$, and the formaldehyde was measured by treating 0.50 ml samples with chromotropic acid as described elsewhere.[24] The reaction mixture contained 1.11 μmoles formaldehyde. Under these conditions 1.00 μmole batyl-alcohol gave 1.06 μmoles formaldehyde. — Identical reaction mixtures obtained with known samples of free glycerol were used as formaldehyde standards.

Partial alkaline hydrolysis of EE–GPE and accompanying lipids. A sample (724 μg P) of Fr. 1/12 was treated with NaOH in the same way as the SPH above, but all solvent volumes were scaled down 12.5-fold. The hydrolysate gave 606 μg water soluble P and 121 μg lipid soluble P. A sample (119 μg P) of the latter fraction was subjected to silicic acid chromatography (Run No. 18; Fig. 4). Fr. 8–11/18, *i.e.* Peak K, (41 μg P) contained AE–GPE and VE–GPE which were contaminated with the "Z-Compounds" (Table 2). Fr. 14–20/18, *i.e.* Peak L, (69 μg P) contained only A–GPE and V–GPE (Table 2).

Mild acid hydrolysis of the mixture of AE–GPE, VE–GPE and "Z-Compounds". A sample (24.9 μg P) of Fr. 8–11/18 was subjected to mild acid treatment in the same way as the mixture of AE–GPC and VE–GPC above. Partition of the hydrolysate gave 23.6 μg lipid soluble P. This fraction was chromatographed on 2.0 g silicic acid (Run No. 19), Fr. 8–11/19, eluted with C–M (6:1), contained AE–GPE (11 μg P), but this preparation was still contaminated by the "Z-Compounds" (Table 2). Fr. 14–18/19 (9.6 μg P), eluted with C containing 20–33 % M, contained pure E–GPE (Table 2).

Mild alkaline hydrolysis of the mixture of AE–GPC and "Z-Compounds". A sample (9 μg P) of Fr. 8–11/19 was treated with NaOH in the same way as the SPH above, but all solvent volumes were scaled down 50-fold, and the reaction was continued for 90 min. Partition of the hydrolysate gave 7 μg lipid soluble P. TLCh. of this stable fraction revealed A–GPE and "Z-Compounds", but AE–GPE had disappeared almost completely.

Mild acid hydrolysis of the mixture of A–GPE and V–GPE. A sample (36.6 μg P) of Fr. 14–20/18 was subjected to mild acid treatment in the same way as the mixture of AE–GPC, and VE–GPC above. Partition of the hydrolysate gave 18.2 μg water soluble P and 19.3 μg lipid soluble P. On TLCh the latter fraction gave only the spot common to all "monochain" derivatives of GPE. It was thus nearly pure A–GPE, but it may have been contaminated with small amounts of cyclic acetal derivatives.[34]

Strong acid hydrolysis of A–GPE. A sample (0.50 μmoles) of the A–GPE described above was subjected to strong acid hydrolysis under the conditions used for the analytical determination of glycerol. When the aqueous phase of the extracted hydrolysate was oxidized with periodate only 0.13 μmoles of apparent glycerol was found, but TLCh. of the C phase of the hydrolysate revealed simple glyceryl ethers, quite as expected.

DISCUSSION

The qualitative composition of the serum phosphatides as revealed in this study is in general agreement with other recent investigations. Phosphatidylcholines, sphingomyelins, lysophosphatidylcholines, and phosphatidylethanolamines have been isolated from serum in many other laboratories,[3–7,9] and the presence of inositides,[10–12,25] ethanolamine and choline plasmalogens,[3,12,13] as well as lysophosphatidylethanolamines [11] has also been already more or less rigidly indicated. However the lysophosphatidylinositols and the alkoxy derivatives of glycerophosphorylcholine and -ethanolamine have probably not been previously observed in serum. The presence of the different alkoxy phosphatides in serum as well as in bovine tissues,[18–20,34] suggests that these lipids are possibly quite widely distributed.

The present investigation does not show whether the lysoderivatives of phosphatidylcholine, -ethanolamine, and -inositol really are native components of serum, for they could be also artificial degradation products of the corresponding diacyl phosphatides or plasmalogens. Phillips,[35] Newman *et al.*[36] and, quite recently, Glomset [37] have shown, however, that lysophosphatidylcholines really are native serum lipids, and very interesting metabolites indeed. In addition Keenan and Hokin [38] have recently isolated lysophosphatidylinositol from pigeon pancreas, and shown that it is easily converted to phosphati-

dylinositol by pancreas and brain homogenates. Therefore I am inclined to think that lysophosphatidylethanolamines and lysophosphatidylinositols might be genuine serum components too.

This opinion can be experimentally argumented as follows: Lysophosphatidylinositols must have been present in Peak D of Fig. 1, for only this can explain the isolation to the different inositol lipids, the lysophosphatidylinositols from Peak D, and the phosphatidylinositols from the other fractions of Fig. 1, by subsequent alumina chromatography. The Peak D material had never been in contact with other adsorbents than silicic acid. Silicic acid may attack the vinylether groups of the plasmalogens,[39] but very likely it does not hydrolyze the acyl ester groups of the phosphatides.[27] This means that if the lysophosphatidylinositols of Peak D were not genuine serum lipids they should be regarded as degradation products of inositol plasmalogens rather than of phosphatidylinositols. However, the presence of any inositol plasmalogens in serum is questionable as the isolated phosphatidylinositols did not absorb any trace of iodine under such conditions [40] where choline and ethanolamine plasmalogens do. Similarly lysophosphatidylethanolamines were already present in Peak C of Fig. 1. If artefacts they too must then be regarded as degradation products of the corresponding plasmalogens rather than of phosphatidylethanolamines. Chromatographic fractionation of the alkali stable fraction of the original serum extract, however, did not reveal sufficiently large amounts of stable ethanolamine phosphatides to account for all the lysophosphatidylethanolamines found. It is a pity that this early experiment of ours was not quite impeccable technically.

I think that the principal observation made during the present study is that the serum phosphatides could be subjected to systematic fractionation and refractionation with several different methods. This principle of multiple fractionation has been more or less neglected in phosphatide work, but it seems that the fear of the general lability of the phosphatides may have been too great.

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