Endogenous phospholipids of swine liver: effect of fat deprivation on molecular species of phosphatidylcholine and phosphatidylethanolamine

J. Michael Shaw[1] **and Nestor R. Bottino**

Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843

Abstract Three 1-yr-old swine and two 2.5-wk-old swine were fed a fat-free diet for 1 month and 5 months, respectively. The hepatic phosphatidylcholine and phosphatidylethanolamine were fractionated by silver ion thin-layer chromatography. A distinctive feature of the chromatographic procedure was the development of the chromatograms at low temperatures: −10°C for phosphatidylcholine and 4°C for phosphatidylethanolamine. The chromatographic fractions were hydrolyzed with phospholipase A_2, and the fatty acids were characterized. Significant concentrations of odd-chain saturated and unsaturated fatty acids were found in the swine deprived of fat for 5 months. The major molecular species of phosphatidylcholine in both groups contained monoenoic fatty acids: 16:0/18:1(n − 9), 18:0/18:1(n − 9), and 18:1(n − 9)/18:1(n − 9). Their concentrations changed only slightly with the diet. The molecular species of phosphatidylethanolamine were more sensitive to dietary changes. In the swine deprived of fat for 1 month, about 50% of the molecular species of phosphatidylethanolamine contained tetraenoic fatty acids: 16:0/20:4(n − 6), 18:0/20:4(n − 6), and 18:1(n − 9)/20:4(n − 6). The phosphatidylethanolamine of animals deprived of fat for 5 months contained only 3% molecular species with tetraenoic acids, 18:0/20:4(n − 6), but 36% molecular species with trienoic acids: 18:0/20:3(n − 9), 18:1(n − 9)/20:3(n − 9), 18:0/19:3(n − 8), 16:0/20:3(n − 9), and 17:0/20:3(n − 9). Doubly unsaturated species, such as 18:1(n − 9)/18:1(n − 9), 18:1(n − 9)/20:3(n − 9), and 18:1(n − 9)/20:4(n − 6), were found in both groups of swine, although their total concentrations were higher in the group deprived of fat for a longer period.

Supplementary key words silver ion thin-layer chromatography · unsaturated phospholipids · phospholipid fatty acid positional distribution · odd-chain (n − 8) fatty acids

Animal tissue phospholipids are complex mixtures of molecules of both endogenous and exogenous origin. Several studies have been made to characterize the effect of dietary fatty acids on the molecular species of animal phospholipids (1–4), but the molecular species compositions of purely endogenous phospholipids are still unknown. The present report describes the molecular species compositions of liver phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from 1-yr-old swine fed a fat-free diet for 1 month and from 2.5-wk-old swine fed a fat-free diet for 5 months. The fatty acids of the group deprived of fat for 5 months are predominantly endogenous, whereas those from the swine deprived of fat for 1 month are a mixture of endogenous and exogenous origin. A comparison of the two groups demonstrates how the diet affects the molecular species of liver phospholipids of swine. A preliminary account of these studies has been presented (5).

MATERIALS AND METHODS

Animal treatment

One group of three Yorkshire–Hampshire swine was raised on a fat-containing diet (Milo corn variety) until approximately 1 yr of age, and then it was placed on a fat-free diet (Ref. 6, diet no. 16) for 1 month (1M group). The two swine in the other group were taken from their mother at 2.5 wk of age and raised on the same fat-free diet for 5 months (5M group). At the end of the fat-free diet periods, the swine were slaughtered and their livers were excised. Hepatic lipids were extracted (7, 8) and pooled according to group. They are designated according to type (i.e., PC or PE) and animal source (5M or 1M); e.g., 5M-PC is phosphatidylcholine from livers of swine fed the fat-free diet for 5 months. Swine fed the fat-containing diet for 5.5 and 13 months were used as controls.

Abbreviations: Ag-TLC, silver ion thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; DCF, 2,7-dichlorofluorescein; EGSS-X, methyl silicone–ethylene glycol succinate polymer; fatty acids are designated by number of carbons:number of double bonds.

[1] Present address: Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pa. 19140.

Chemicals and solvents

Chemicals and solvents were of reagent grade; the solvents, chloroform, methanol, *n*-propanol, diethyl ether, hexane, and benzene, were redistilled before use in TLC or extraction. Large volumes (300–500 ml) of chloroform, methanol, diethyl ether, and hexane were evaporated to less than 1 ml and monitored by GLC for possible contamination. Silica gel H or HR (Brinkmann, Westbury, N.Y.) were used in the TLC fractionations. Silica gel H was purified by washing approximately 200–300 g with 1000 ml of chloroform–methanol–glacial acetic acid–water 50:39:1:10 (v/v). The washed silica gel H was dried at 110°C for 12–24 hr and ground with mortar and pestle. Silica gel H was used only for silver nitrate TLC, whereas silica gel HR was used without further purification for other TLC fractionations. Cation exchange resin (AG50-XB, Bio-Rad Laboratories, Richmond, Calif.) was washed with 15–20 ml of chloroform–methanol–glacial acetic acid–water 50:39:1:10, and the washings were monitored for contamination by GLC. The 2,7-dichlorofluorescein (DCF) was purified by the method used by Parker and Peterson (9).

Extraction, isolation, and purification of PC and PE

After extraction of tissue lipids by the method of Folch, Lees, and Sloane Stanley (7) or that of Bligh and Dyer (8), the total lipids were placed in a slurry of silicic acid in chloroform. Neutral lipids were extracted from the slurry with chloroform; phospholipids were extracted with either methanol or chloroform–methanol–glacial acetic acid–water 50:39:1:10.

For the fractionation of total phospholipids, it was necessary in most cases to utilize two TLC procedures. The two-step technique described by Neskovic and Kostic (10) was utilized first, followed by elution of PC and PE spots from the gel (11) and development on a second thin-layer plate with chloroform–methanol–glacial acetic acid–water 130:86:2:6 (12). The purified PC and PE gave single spots in various one-dimensional and two-dimensional TLC systems. Cochromatography of the purified PC and PE with known standards (Supelco, Bellefonte, Pa., and Applied Science Laboratories, State College, Pa.) and the appropriate reactions with ninhydrin, Dragendorf (13), and Dittmer-Lester (14) reagents were used as checks. Purity, as measured by phosphorus assay (15), was 99% for PC and 98% for PE.

Quantitation and recovery of phospholipids

Phospholipids were determined by the phosphorus assay of Chen, Toribara, and Warner (15) after elution from the silica gel. The assay was modified slightly in that digestion was carried out in a heating block with time periods suggested by Bartlett (16). The fractions of PC and PE were quantitatively eluted from the silica gel with the solvent system of Arvidson (11). Recovery from a mixture of silica gel, silver nitrate, and cation exchange resin with the same solvent system and extraction procedure was 100% for PC and 95% for PE.

Ag-TLC

The preparation and heating of thin layers were as follows. A silver nitrate–silica gel slurry was prepared by vigorously mixing 55 g of purified silica gel H and 7.5 g of silver nitrate dissolved in 124 ml of distilled water in a stainless steel blender. The slurry was spread in 0.75-mm layers on three glass plates (400 × 200 mm) and left to air-dry for 5–12 hr. The air-dried thin layers were placed in an oven at 195°C for 2.5 hr and removed from the oven after a 45-min to 1-hr cooling period. Finally, the thin layers were transferred to a desiccator containing P_2O_5 and cooled to room temperature.

The developing solvent system consisted of a mixture of chloroform–methanol–*n*-propanol–0.5% acetic acid in water 220:120:20:28, which was placed in a 270 × 75 × 250 mm TLC tank. A few grains of butylated hydroxytoluene were also added, and a large circular wick of blotting paper approximately 40 cm in height was placed inside the tank. To accommodate the long 200 × 400 mm plates, an additional TLC tank was next inverted atop the tank containing the solvent system and large wick. Teflon tape was used to seal the two tanks. The prepared tanks were placed in a −10°C freezer or in a 4°C cold room overnight for temperature equilibration.

PC or PE (100 μg of P) was spotted under nitrogen in bands 3–4 cm long. The thin layers containing PE were placed in the solvent system at 4°C immediately after spotting, without any previous equilibration. Thin layers containing PC were first fixed in a sandwich style with Teflon strips for keeping the layers apart. Thus, two plates in sandwich were placed in the −10°C freezer for development. The time of development was approximately 10–11 hr for PE and 14–16 hr for PC. After development, the plates were brought to room temperature under N_2 and sprayed with 0.2% DCF in methanol. The fractions were located under UV light and scraped from the plate.

Elution and extraction of PC and PE from silver ion-containing gel were accomplished by the technique of Arvidson (11) plus a treatment with cation exchange resin to remove the remaining silver (17).

Phospholipase A_2 hydrolysis

Phospholipase A_2 (king cobra venom purchased from Ross Allen Reptile Institute, Silver Springs, Fla.) was partially autodigested and then extracted with diethyl ether, as indicated by Kuksis et al. (18). Phospholipase hydrolyses of total PC and PE and their Ag-TLC fractions were conducted, as described by Nutter and Privett (19), on 5–10-mg samples for 2.5–3 hr. In all cases, more

than one Ag-TLC run was necessary to collect 5–10 mg of the fractions. The products of phospholipase hydrolysis were fractionated by two-step TLC (20), ammonia being used after the first development to neutralize the acetic acid remaining in the thin layer. Free fatty acids were eluted from the silica gel with diethyl ether followed by chloroform. Lysophospholipids were eluted with chloroform–methanol–acetic acid–water 50:39:1:10 and further extracted, as described by Arvidson (11). The percentage recoveries of various lyso derivatives were 94% for egg lyso-PC, 91% for swine lyso-PC, and greater than 99% for swine lyso-PE. The extent of hydrolysis in the chosen time period was determined by estimating phosphorus in the hydrolyzed and unhydrolyzed materials. For example, the degrees of hydrolysis of fraction III of 5M-PC and fraction III–III′ of 5M-PE were 99.7% and 94.8%, respectively.

Identification of fatty acid components

Methyl esters were prepared by the method of Metcalfe, Schmitz, and Pelka (21), modified by using approximately 10% boron trifluoride (BF_3) in methanol (prepared by bubbling BF_3 into redistilled methanol), with a reflux time of 15 min after the addition of the BF_3 solution. Methyl esters were purified by TLC, using benzene as the solvent system. The purified methyl esters were dissolved in carbon disulfide and analyzed in a Beckman GC-5 gas chromatograph with a dual hydrogen flame detector. A 6-ft stainless steel column, ⅛ inch OD, was packed with 10% methyl silicone–ethylene glycol succinate polymer (EGSS-X) on 100–120 mesh Gas-Chrom P (Applied Science Laboratories). The instrument was periodically checked for area percentage–weight percentage correlation using NIH standard mixtures. Weight percentage values for fatty esters were determined from the chromatograms by multiplying peak height times width at half height. The weight percentages were converted to mole percentages with no additional correction factors applied.

The possibility of methoxy artifact formation using BF_3 methanol as described by other investigators (22, 23) was not confirmed when using the present technique. For example, oleic acid (purchased from Hormel Institute, Austin, Minn.) was converted to methyl ester, and no artifact formation was apparent when analyzing the methyl esters by GLC.

Fatty acids were identified by comparing their relative retention times with those of standards and by semilog plots of relative retention times vs. carbon numbers.

Fatty acid methyl esters of all 1M-PC fractions, of most fractions of the 5M group, and of their phospholipase hydrolysis products were also studied by GLC after hydrogenation (24). In addition, the fatty acid methyl esters of some fractions of the 5M group and their phospholipase hydrolysis products were fractionated by Ag-TLC (25) and examined by GLC. The assignment of double bond positions is based on GLC data and is in some cases only tentative.

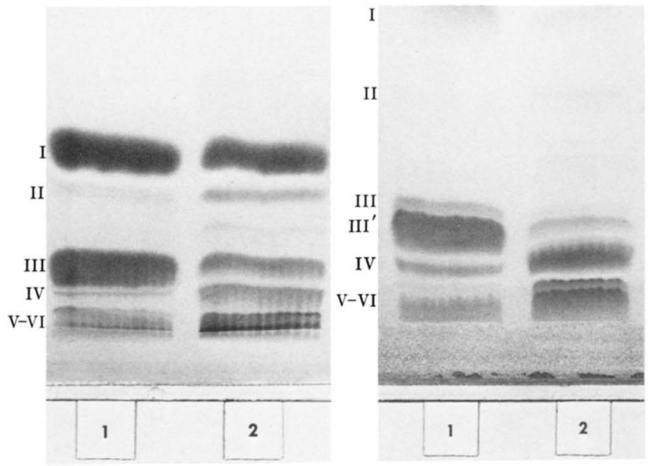

Fig. 1. Ag-TLC fractionation of PC (*left*) and PE (*right*). Lane *1*, liver phospholipids of swine fed a fat-free diet for 5 mo; lane *2*, liver phospholipids of swine fed a fat-free diet for 1 mo. Thin layers 0.75 mm thick, containing 12% (w/w) silver nitrate–silica gel, were heated at 195°C for 2.5 hr. Sample size was approximately 120 μg of phosphorus for PC and 100 μg of phosphorus for PE, applied in 3–4-cm bands. Development was performed with the solvent system chloroform–methanol–*n*-propanol–0.5% acetic acid in water 220:120:20:28. PC was developed at −10°C for 14–16 hr. PE was developed at 4°C for 11–12 hr. Visualization of fractions was accomplished by spraying with 20% (w/v) aqueous solution of $(NH_4)_2SO_4$ followed by charring at 180–200°C (47).

RESULTS AND DISCUSSION

Ag-TLC of intact phospholipids

Fig. 1 illustrates the type of results achieved with the Ag-TLC technique in the separation of intact phospholipids. Important points in the successful fractionation of these phospholipids are: (*a*) High-temperature (195°C for 2.5 hr) treatment of the thin layers before chromatography. (*b*) Medium-range concentration of silver nitrate in silica gel, 10–15%. (*c*) A solvent system for chromatographic development composed of chloroform–methanol–*n*-propanol–0.5% acetic acid in water 220:120:20:28. This system provided the most adequate resolution. The inclusion of *n*-propanol or other alcohols, such as *n*-butanol or *tert*-butanol, made the bands after fractionation more distinct and compact and reduced tailing. (*d*) Low temperature of chromatographic development. The most adequate fractionation of PE was obtained at 4°C, whereas PC was optimally fractionated at −10°C. We feel that both the different degree of unsaturation and the nature of the polar portion of PC and PE are responsible for the different optimum temperatures. The use of low temperature of development in Ag-TLC fractionation of phospholipids has been previously reported by Shaw and Bottino

TABLE 1. Major fatty acids of phospholipids from swine liver[a]

Fatty Acid	Fat-free Diet: Age 5.5 mo Diet 5 mo PC	Fat-free Diet: Age 5.5 mo Diet 5 mo PE	Fat-free Diet: Age 13 mo Diet 1 mo PC	Fat-free Diet: Age 13 mo Diet 1 mo PE	Fat-containing Diet: Age 5.5 mo PC	Fat-containing Diet: Age 5.5 mo PE	Fat-containing Diet: Age 13 mo PC	Fat-containing Diet: Age 13 mo PE	Total Dietary Lipids
					mole %				
16:0	14.4	6.6	22.3	11.4	20.2	8.6	26.2	11.2	17.0
17:0	3.0	4.3	0.9	1.1	0.7	1.2	1.1	0.9	0.1
18:0	17.9	23.0	20.1	27.3	26.0	37.9	30.1	37.5	1.9
16:1(n − 7)	4.8	2.0	4.3	2.1	1.2	0.5	1.3	tr	1.0
17:1(n − 8)	3.2	2.4	0.4	0.6	0.3	tr	0.3		0.4
18:1(n − 9)	36.6	25.0	30.5	13.9	18.3	8.1	14.6	5.6	28.8
18:2(n − 9)	1.4	1.7							
18:2(n − 6)			2.9	2.5	13.6	7.0	12.7	5.5	46.8
18:3(n − 3)					0.4	0.4	0.3	0.3	2.6
19:3(n − 8)	2.7	4.7	0.2						
20:3(n − 9)	10.1	18.8	4.9	3.4					
20:4(n − 6)	0.8	5.4	7.1	26.2	12.3	26.8	7.8	26.3	
22:5(n − 3)	0.1	0.3	1.2	2.4	1.4	1.8	1.3	4.5	
22:6(n − 3)	0.1	0.3	1.8	4.9	1.1	2.2	0.7	4.1	

[a] Only those fatty acids present at a level of 1% or more are included.

(5) and Luthra and Sheltawy (26). The following factors might have contributed to improved separations at low temperatures. The partition coefficient increases with decreasing temperature (27). Adsorptivity increases at lower temperatures and small differences in relative adsorption of two solutes may be amplified so as to allow their separation (28). An example is the separation by Clements (29) of saturated from olefinic terpenes on silicic acid without silver nitrate at −78.5°C. Furthermore, low temperature of development might "freeze in" excited molecular states during chromatography (30). An adverse effect of low temperature of development is the increase in the viscosity of the stationary phase, causing a decreased diffusion of solutes in the liquid phase (27). However, the higher viscosity of the solvents lengthens the time of development and allows a more intimate contact between the mobile and stationary phases. Finally, another beneficial factor in low-temperature separations by Ag-TLC is the increased stability of the silver ion–olefin complexes (31).

Reproducibility

The reproducibility of the technique was proved in two ways. (*1*) The fatty acid compositions of equivalent fractions did not differ greatly from one chromatographic run to another. Paired *t* tests between duplicate determinations showed nonsignificant differences at the 5% level for either the large or the small fractions. (*2*) The fatty acid compositions of the unfractionated phospholipids compared well with those recalculated from the proportions of the fractions and the fatty acid compositions of the fractions. The paired *t* test between the original and the recalculated data showed nonsignificant differences at the 5% level. The excellent recovery of polyunsaturated fatty acids is attributed to complete extraction of all silica gel areas and reduced autoxidation of the more unsaturated fatty acids at the low temperature of Ag-TLC development. The application of the technique to the fractionation of highly unsaturated PC and PE of sei whale liver has been reported recently (32).

Fatty acid patterns

The literature abounds in demonstrations of how feeding a fat-deficient diet to animals results in a gradual replacement of the typical exogenous fatty acids of the linoleic (n − 6) and linolenic (n − 3) families by endogenous fatty acids of the oleic (n − 9) and palmitoleic (n − 7) families (see review, Ref. 33). These effects, although demonstrated primarily in rats (34), have been reported also in swine liver total lipids (35) and swine serum phospholipids (36). In the present work, swine deprived of fat for either 1 month or 5 months showed the expected replacement of exogenous by endogenous fatty acids in PC and PE of liver (**Table 1** and **Figs. 2** and **3**). In PC, the (n − 3) plus (n − 6) fatty acids declined from about 25–30% in the animals fed fat to 16% in those fed no fat for 1 month and to 2% in those deprived of fat for 5 months. In PE, the (n − 3) plus (n − 6) fatty acids were 44% on a fat diet, 39% after 1 month on the fat-free diet, and 6% after 5 months of fat deprivation. The retention of 2% and 6% exogenous fatty acids in 5M-PC and 5M-PE, respectively, after 5 months of fat deficiency reflects the low turnover rates of certain polyunsaturated molecular species (37).

The Ag-TLC fractionation technique increases the possibility of finding fatty acids that otherwise may remain undetected in the unfractionated samples. In both groups of swine, members of the linoleic acid family were found that suggested the following sequence of biosynthesis:

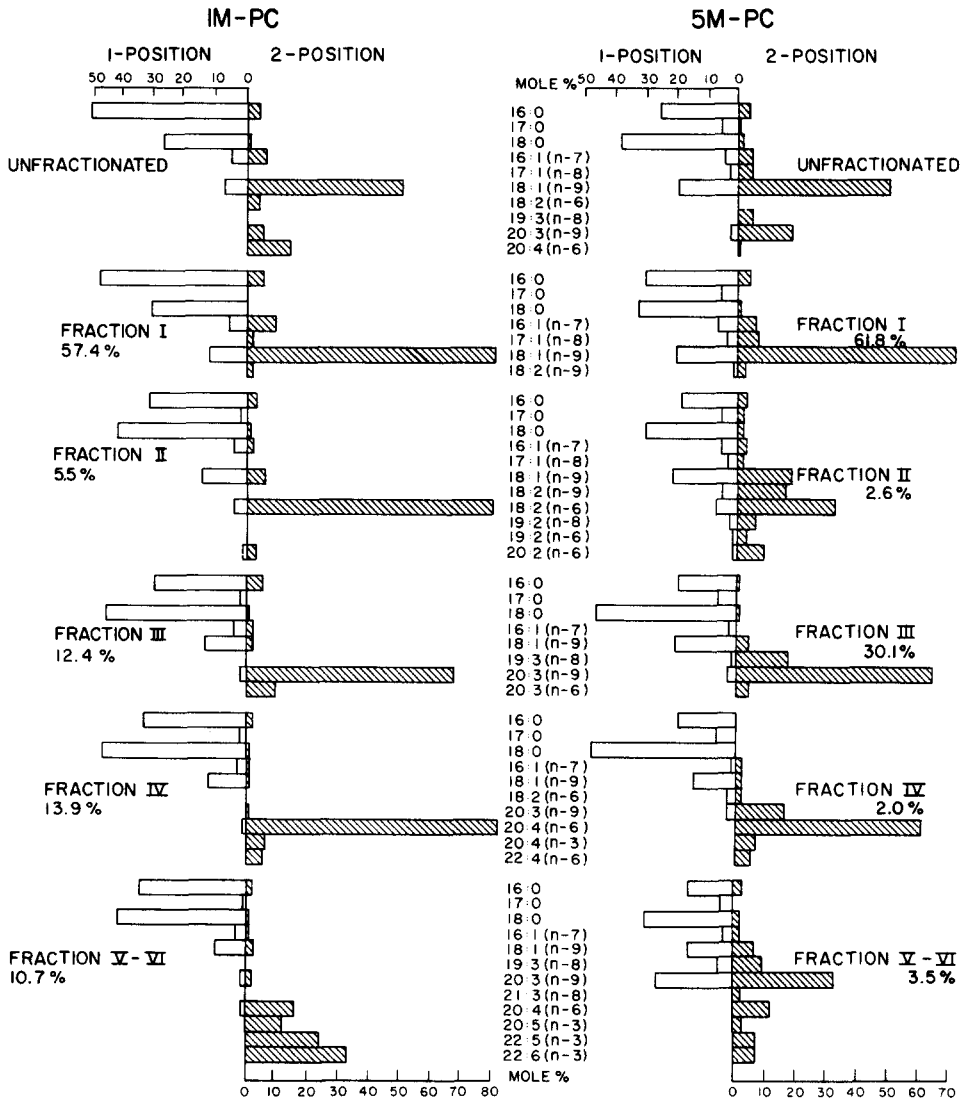

Fig. 2. Positional distribution of the fatty acids of swine liver PC. PC from swine fed a fat-free diet for 1 mo (1M-PC) and 5 mo (5M-PC) was fractionated by Ag-TLC and the fractions were hydrolyzed with phospholipase A_2. The figure shows the major fatty acids (more than 2%) of the resultant lyso-PC (1-position) and free fatty acids (2-position). The 19:2(n − 6) and 22:4(n − 6) acids are only tentatively identified. Complete fatty acid compositions are available from N.R.B. upon request.

18:2(n − 6)→18:3(n − 6)→20:3(n − 6)→20:4(n − 6)→22:5(n − 6). From the linolenic acid family the following members were identified: 18:3(n − 3)→20:4(n − 3)→20:5(n − 3)→22:5(n − 3)→22:6(n − 3). These intermediates coincide with those characterized in rat liver by Mead (38) and Klenk (39). From the typical endogenous families, only 16:1(n − 7) was found from the palmitoleic family, whereas the oleic family was represented by 18:1(n − 9)→18:2(n − 9)→20:2(n − 9)→20:3(n − 9)→22:3(n − 9). This sequence has been reported in the liver of rats fed essential fatty acid-deficient diets (33). In addition to the four "classic families," the hepatic phospholipids of swine in acute fat deprivation (5M-PC and 5M-PE) contained fatty acids of 17, 19, and 21 carbon atoms with 0 to 3 double bonds. On the basis of the fatty acids found in 5M-PC (Fig. 2) and 5M-PE (Fig. 3), the following sequence of biosynthesis is suggested: 17:0→171(n − 8)→19:1(n − 8)→19:2(n − 8)→19:3(n − 8)→21:3(n − 8). The location of the double bonds is tentative and the sequential order is speculative. Substantial amounts of 17:0 and 17:1 fatty acids were found in the liver triglycerides of the same swine (40). Schlenk and Sand (41) have reported 15:0, 17:0, 17:1(n − 8), and 19:3(n − 8) in the livers of rats on fat-free diets.

Fatty acid composition and positional distribution

Examination of the data in Figs. 2 and 3 indicates that in both PC and PE fatty acids are distributed among Ag-TLC fractions according to two general patterns. Significant concentrations of saturated (25–47%) and monoenoic

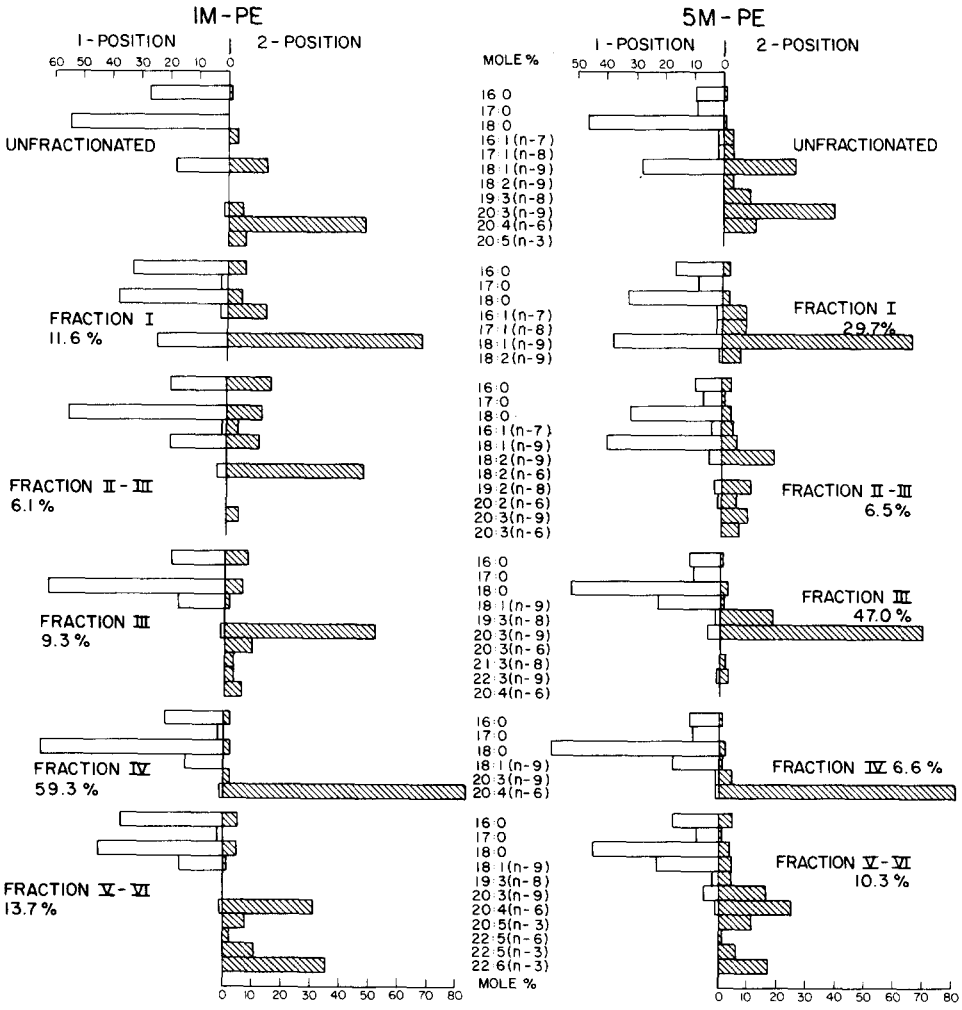

Fig. 3. Positional distribution of the fatty acids of swine liver PE. PE from swine fed a fat-free diet for 1 mo (1M-PE) and 5 mo (5M-PE) was fractionated by Ag-TLC and the fractions were hydrolyzed with phospholipase A_2. The figure shows the major fatty acids (more than 2%) of the resultant lyso-PE (1-position) and free fatty acids (2-position). The 22:3(n − 6) fatty acid is only tentatively identified. Fraction III of 5M-PE includes fractions III and III′ of Fig. 1 (*right*).

(7–64%) fatty acids are present in all fractions. The polyenoic fatty acids are found predominantly in one particular fraction; for example, trienoic acids in fraction III[2] and tetraenoic acids in fraction IV. An exception is fraction V–VI, which contains two or more types of polyunsaturated fatty acids. Saturated and monoenoic fatty acids account for approximately 50% of the fatty acids in each Ag-TLC fraction, with the exception of fraction I of every group and fraction V–VI of 5M–PC. Polyenoic fatty acids represent the remaining 50% of fractions II, III, and IV. These percentage values correlate well with the positional distributions of the fatty acids, as determined by phospholipase A_2 hydrolysis. Saturated fatty acids occupied preferentially the 1-position and polyenoic fatty acids the 2-position; monoenoic fatty acids varied in positional distribution, the position depending on the partner. When associated with saturated acids, monoenes occupied preferentially the 2-position; when associated with polyenes, they esterified the 1-position.

Phospholipid molecular species and effect of diet

The proportions of molecular species of swine liver phospholipids were calculated from Ag-TLC, GLC, and positional distribution data, as described by van Golde and van Deenen (2). Such a calculation provides a good estimate of the individual molecular species when there is a strong predominance of one fatty acid in one of the two positions of the phospholipids. When there is no strong positional predominance, the calculation requires certain assumptions.

In Figs. 2 and 3 there are many examples of large concentrations of only one fatty acid at the 2-position. For ex-

[2] The roman numeral assigned to each Ag-TLC fraction denotes the number of double bonds of its major unsaturated fatty acids and not the total number of double bonds in the fraction.

ample, fraction IV of 1M-PE (Fig. 2) contains 93% tetraenoic fatty acids at the 2-position, and 82% of the tetraenoic acids is 20:4(n − 6). Therefore, the major molecular species of fraction IV can be easily calculated by the method of van Golde and van Deenen (2). On the other hand, fraction II of 1M-PE, for example (Fig. 3), contains 15% 16:0, 12% 18:0, 11% 18:1(n − 9), and 47% 18:2(n − 6) at the 2-position. In this case, to calculate molecular species we have assumed a random association of 1-positioned fatty acids with 2-positioned acids. The following is an example. The calculations required to obtain the proportion of the 18:0/20:4(n − 6) (18:0 at *sn*-1-position/20:4[n − 6] at *sn*-2-position) species in fraction IV of 1M-PE (Table 2) are:

% 1-stearoyl PE = (% 18:0 in 1-position) ×
(% fraction IV)/100 = 63.1 × 59.3/100 = 37.4
% 18:0/20:4(n − 6) species = (% stearoyl PE) ×
(% 20:4 in 2-position)/100 = 37.4 × 82.8/100 = 31.0

The calculated data **(Table 2)** illustrate the typical replacement of species containing exogenous linoleic, arachidonic, and (n − 3) acids by species containing endogenous (n − 9) oleic and eicosatrienoic acids. In PC, the monoenoic species, mainly 16:0/18:1(n − 9), constitute 35–40% of all molecular species regardless of the degree of fat deprivation. On the other hand, the molecular species of PC that contain exogenous (n − 6) and (n − 3) acids change drastically with the dietary treatment, from 13% in 1M-PC to none in 5M-PC. This decrease in the exogenous families (n − 6) and (n − 3) is compensated for by an increase in trienoic acid species from 6% in 1M-PC to 19% in 5M-PC.

To our knowledge, no previous studies are available on the effect of diet on the molecular species of PE. The data in Table 2 show that the molecular species composition of PE is quite different from that of PC and that the effects of a diet without fat are more marked on PE than on PC.

Approximately 50% of the molecular species of 1M-PE are tetraenoic, mainly 18:0/20:4(n − 6), 31%; 16:0/20:4(n − 6), 10%; and 18:1(n −9)/20:4(n − 6), 6%. On the other hand, the predominant species in 5M-PE contain trienoic acids (36%) and monoenoic acids (18%): 18:0/20:3(n − 9), 17%; 18:1(n − 9)/20:3(n − 9), 7%; 18:1(n − 9)/18:1(n − 9), 7%; 18:0/18:1(n − 9), 6%. Comparing 1M-PE with 5M-PE, one can observe a decline of tetraenoic species from 48% in 1M-PE to 4% in 5M-PE and of penta- and hexaenoic species from 5% in 1M-PE to none in 5M-PE. Concomitantly, the trienoic species increase from 3% in 1M-PE to 36% in 5M-PE, the monoenoic species from 7% to 18%, and the dienoic species from 1% to 7%. In either dietary treatment, PE is more unsaturated than PC, with monounsaturated species prevailing in PC and polyunsaturated species in PE. In both phospholipid classes the result of fat deprivation is a shift in synthesis in favor of trienoic molecular species such as saturated/trienoic and monoenoic/trienoic rather than tetraenoic molecular species such as saturated/tetra-

TABLE 2. Major molecular species[a] of PC and PE from livers of swine fed a fat-free diet for various periods of time

Molecular Species	1M-PC[b]	5M-PC[c]	1M-PE[b]	5M-PE[c]
		mole %		
Fraction I				
16:0/16:0[d]	1.5			
18:0/16:0	1.0			
16:0/16:1(n − 7)	2.3	1.2		
16:0/17:1(n − 8)		1.2		
16:0/18:1(n − 9)	22.2	13.2	2.5	3.0
17:0/18:1(n − 9)		2.4		1.6
18:0/16:1(n − 7)	1.5	1.2		
18:0/17:1(n − 8)		1.3		
18:0/18:1(n − 9)	14.6	14.1	2.9	6.3
16:1(n − 7)/18:1(n − 9)[e]	2.6	2.7		
17:1(n − 8)/18:1(n − 9)[e]		1.3		
18:1(n − 9)/18:1(n − 9)[e]	5.6	8.9	1.9	7.1
Total	51.3	47.5	7.3	18.0
Fraction II				
16:0/18:2(n − 6)	1.4			
18:0/18:2(n − 6)	1.8		1.2	
18:1(n − 9)/18:1(n − 9)				1.6
18:1(n − 9)/18:2(n − 9)[e]				1.6
18:1(n − 9)/18:2(n − 6)[e]				2.0
18:1(n − 9)/19:2(n − 8)[e]				1.1
18:1(n − 9)/20:3(n − 9)[e]				1.0
Total	3.2		1.2	7.3
Fraction III[f]				
16:0/20:3(n − 9)	2.2	3.5		3.1
17:0/20:3(n − 9)		1.1		2.9
18:0/19:3(n − 8)		2.2		4.4
18:0/20:3(n − 9)	3.4	8.6	3.0	16.5
18:1(n − 9)/19:3(n − 8)[e]				1.9
18:1(n − 9)/20:3(n − 9)[e]		3.8		6.9
Total	5.6	19.2	3.0	35.7
Fraction IV				
16:0/20:4(n − 6)	3.4		9.6	
18:0/20:4(n − 6)	5.4		31.0	3.0
18:0/22:4(n − 6)			1.1	
18:0/22:5(n − 3)			1.1	
18:1(n − 9)/20:4(n − 6)[e]	1.4		6.3	
Total	10.2		49.1	3.0
Fraction V–VI				
16:0/20:4(n − 6)			1.5	
16:0/22:6(n − 3)	1.2		1.6	
18:0/20:4(n − 6)			1.8	1.1
18:0/22:5(n − 3)	1.1			
18:0/22:6(n − 3)	1.5		2.0	
Total	3.8		6.9	1.1

[a] For method of calculation, see text. Only those molecular species present in amounts greater than 1% are shown.
[b] Swine were fed a fat-containing diet for approximately 1 yr after weaning and then fed a fat-free diet for 1 mo (1M group).
[c] Swine were fed a fat-free diet for 5 mo after weaning (5M group).
[d] Fatty acid at *sn*-1-position/fatty acid at *sn*-2-position.
[e] Molecular species containing two unsaturated fatty acids.
[f] Fraction III of 5M-PE includes fractions III and III′ of Fig. 1 (*right*).

enoic. Consequently, the changes in unsaturation produced by fat deprivation are more drastic in PE than in PC.

In Table 2 are shown the molecular species of PC and PE that contain unsaturated fatty acids at both positions. The more abundant species are 16:1(n − 7)/18:1(n − 9), 18:1(n − 9)/18:1(n −9), 18:1(n − 9)/20:3(n − 9), and 18:1(n − 9)/20:4(n − 6). The proportions of doubly unsaturated molecular species increase from 10% of the total species in 1M-PC to 17% in 5M-PC and from 8% in 1M-PE to 23% in 5M-PE.

The presence of relatively high concentrations of molecular species with two unsaturated fatty acids in the phospholipids of the animals on acute fat deprivation (5M group) may have some relation to the increase in membrane permeability usually found under these conditions (33), because Demel, Guerts van Kessel, and van Deenen (42) have shown that liposomes made of doubly unsaturated phosphatidylcholines exhibit an enhanced permeability to glucose, glycerol, and erythritol.

Chain positional isomerism and Ag-TLC fractionation of phospholipids

Fig. 1 shows the presence in 5M-PE of two fractions carrying trienoic acid, fractions III and III′, which exhibit different migration rates on Ag-TLC (R_F III = 0.17 and R_F III′ = 0.13) despite having an equal degree of unsaturation. Fraction III′ is about ten times larger (43%) than fraction III (4%). Both the types and the concentrations of saturated and monoenoic fatty acids are essentially the same in the two fractions. The trienoic acids are also equivalent in quantity, 47% in fraction III and 49% in fraction III′. However, the nature of the trienoic acids differs between fractions: fraction III contains 17% 22:3(n − 9), 14% 21:3(n − 8), and 12% 20:3(n − 6), whereas fraction III′ contains 39% 20:3(n − 9) and 10% 19:3(n − 8). In other words, the trienoic acids in the faster-moving fraction, fraction III, are Δ7,10,13 and Δ8,11,14 whereas those in the slower-moving fraction are Δ5,8,11. Because these trienoic acids are esterified preferentially to the 2-position (Fig. 3), the only differences between the molecular species in fractions III and III′ are in (*a*) fatty acid chain length and (*b*) location of the double bonds on the chains. Wessels and Rajagopal (43) have shown that fatty acid chain length is not a significant factor in the separation of triglycerides by Ag-TLC. Instead, the distance between the ester linkage and the first double bond of the esterified fatty acid is important. Therefore, it is possible that the migration of phospholipids on Ag-TLC systems might depend not only on their degree of unsaturation but also on the relative positions of the double bonds along the fatty acyl chains. The closer the double bond systems are to the ester bond, the stronger the interaction between the phospholipid molecule and the silver ion, and the slower the migration. The effect seems to be analogous to that reported by Morris, Wharry, and Hammond (31, 44) and by Gunstone, Ismail, and Lie Ken Jie (45, 46) on the migration of unsaturated fatty acid methyl esters on Ag-TLC.

This work was supported in part by NIH grant AM 06011. It was done by J. M. Shaw in partial fulfillment of the requirements for the degree of Master of Science in biochemistry.

Manuscript received 15 December 1972, in revised form 29 May 1973, and in rerevised form 14 January 1974; accepted 1 March 1974.

REFERENCES

1. Van Golde, L. M. G., R. F. A. Zwaal, and L. L. M. van Deenen. 1965. Studies on the molecular species of lecithin from rat liver. *Proc. Kon. Ned. Akad. Wetensch. Ser. B.* **68:** 255–265.
2. Van Golde, L. M. G., and L. L. M. van Deenen. 1966. The effect of dietary fat on the molecular species of lecithin from rat liver. *Biochim. Biophys. Acta.* **125:** 496–509.
3. Van Golde, L. M. G., W. A. Pieterson, and L. L. M. van Deenen. 1968. Alterations in the molecular species of rat liver lecithin by corn-oil feeding to essential fatty acid-deficient rats as a function of time. *Biochim. Biophys. Acta.* **152:** 84–95.
4. Yurkowski, M., and B. L. Walker. 1971. The molecular species of mucosal phosphatidyl cholines from the small intestine of normal and essential fatty acid-deficient rats. *Biochim. Biophys. Acta.* **231:** 145–152.
5. Shaw, J. M., and N. R. Bottino. 1971. Silver nitrate-thin-layer chromatography of phospholipids. Separation of pig and shrimp phosphatidylcholines and phosphatidylethanolamines. *American Oil Chemists' Society* 62nd Annual Meeting, Houston, Texas.
6. Hill, E. G., E. L. Warmanen, C. L. Silbernick, and R. T. Holman. 1961. Essential fatty acid nutrition in swine. I. Linoleate requirement estimated from triene:tetraene ratio of tissue lipids. *J. Nutr.* **74:** 335–341.
7. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.
8. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
9. Parker, F., and N. F. Peterson. 1965. Quantitative analysis of phospholipids and phospholipid fatty acids from silica gel thin-layer chromatograms. *J. Lipid Res.* **6:** 455–460.
10. Neskovic, N. M., and D. M. Kostic. 1968. Quantitative analysis of rat liver phospholipids by a two-step thin-layer chromatographic procedure. *J. Chromatogr.* **35:** 297–300.
11. Arvidson, G. A. E. 1968. Structural and metabolic heterogeneity of rat liver glycerophosphatides. *Eur. J. Biochem.* **4:** 478–486.
12. Owens, K. 1966. A two-dimensional thin-layer chromatographic procedure for the estimation of plasmalogens. *Biochem. J.* **100:** 354–361.
13. Stahl, E., ed. 1969. Thin-Layer Chromatography: A Laboratory Handbook. 2nd ed. Springer-Verlag, New York.
14. Dittmer, J. C., and R. L. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* **5:** 126–127.

15. Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* **28:** 1756–1758.
16. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
17. Kyriakides, E. C., and J. A. Balint. 1968. Quantitative recovery of lecithins after argentation chromatography on thin layers. *J. Lipid Res.* **9:** 142–143.
18. Kuksis, A., L. Marai, W. C. Breckenridge, D. A. Gornall, and O. Stachnyk. 1968. Molecular species of lecithins of some functionally distinct rat tissues. *Can. J. Physiol. Pharmacol.* **46:** 511–524.
19. Nutter, L. J., and O. S. Privett. 1966. Phospholipase A properties of several snake venom preparations. *Lipids.* **1:** 258–262.
20. Johnston, J. M., G. A. Rao, P. A. Lowe, and B. E. Schwarz. 1967. The nature of the stimulatory role of the supernatant fraction on triglyceride synthesis by the α-glycerophosphate pathway. *Lipids.* **2:** 14–20.
21. Metcalfe, L. D., A. A. Schmitz, and J. R. Pelka. 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Anal. Chem.* **38:** 514–515.
22. Lough, A. K. 1964. The production of methoxy-substituted fatty acids as artifacts during the esterification of unsaturated fatty acids with methanol containing boron trifluoride. *Biochem. J.* **90:** 4C–5C.
23. Fulk, W. K., and M. S. Shorb. 1970. Production of an artifact during methanolysis of lipids by boron trifluoride–methanol. *J. Lipid Res.* **11:** 276–277.
24. Anderson, R. E., M. B. Maude, and G. L. Feldman. 1969. Lipids of ocular tissues. I. The phospholipids of mature rabbit and bovine lens. *Biochim. Biophys. Acta.* **187:** 345–353.
25. Privett, O. S., and M. L. Blank. 1964. Studies on the metabolism of linoelaidic acid in the essential fatty acid-deficient rat. *J. Amer. Oil Chem. Soc.* **41:** 292–297.
26. Luthra, M. G., and A. Sheltawy. 1972. The fractionation of phosphatidylinositol into molecular species by thin-layer chromatography on silver nitrate-impregnated silica gel. *Biochem. J.* **126:** 1231–1239.
27. Weissberger, A., and H. G. Cassidy. 1957. Fundamentals of Chromatography. Interscience, New York. 91.
28. Snyder, L. R. 1968. Principles of Adsorption Chromatography. Dekker, New York. 340–342.
29. Clements, R. L. 1958. Low-temperature chromatography as a means for separating terpene hydrocarbons. *Science.* **128:** 899–900.
30. Stahl, E. 1964. Gradient and low-temperature thin-layer chromatography. *Angew. Chem. (Int. Ed.)* **3:** 784–791.
31. Morris, L. J., D. M. Wharry, and E. W. Hammond. 1967. Chromatographic behaviour of isomeric long-chain aliphatic compounds. II. Argentation thin-layer chromatography of isomeric octadecenoates. *J. Chromatogr.* **31:** 69–76.
32. Bottino, N. R. 1972. Phospholipids of antarctic sei whale liver. Fractionation by silver-nitrate thin-layer chromatography. American Oil Chemists' Society 46th Fall Meeting, Ottawa, Canada.
33. Holman, R. T. 1968. Essential fatty acid deficiency. *Progr. Chem. Fats Other Lipids.* **9(2):** 275–348.
34. Klenk, E., and K. Oette. 1960. Über die Natur der in den Leberphosphatiden auftretenden C_{20}- und C_{22}-Polyensäuren bei Verabreichung von Linol- und Linolensäure an fettfrei ernährte Ratten. *Hoppe Seyler's Z. Physiol. Chem.* **318:** 86–99.
35. Leat, W. M. F. 1962. Studies on pig diets containing different amounts of linoleic acid. *Brit. J. Nutr.* **16:** 559–569.
36. Leat, W. M. F. 1964. Serum phospholipids of pigs given different amounts of linoleic acid. I. Fatty acid composition of the kephalin, lecithin, lysolecithin and sphingomyelin fractions. *Biochem. J.* **91:** 437–443.
37. Holub, B. J. and A. Kuksis. 1971. Structural and metabolic interrelationships among glycerophosphatides of rat liver in vivo. *Can. J. Biochem.* **49:** 1347–1356.
38. Mead, J. F. 1968. The metabolism of the polyunsaturated fatty acids. *Progr. Chem. Fats Other Lipids.* **9(2):** 161–192.
39. Klenk, E. 1965. The metabolism of polyenoic fatty acids. *Adv. Lipid Res.* **3:** 1–23.
40. Anderson, R. E., N. R. Bottino, L. J. Cook, and R. Reiser. 1970. Animal endogenous triglycerides. III. Swine, rat and chicken liver: comparison with adipose tissue. *Lipids.* **5:** 171–179.
41. Schlenk, H., and D. M. Sand. 1967. A new group of essential fatty acids and their comparison with other polyenoic fatty acids. *Biochim. Biophys. Acta.* **144:** 305–320.
42. Demel, R. A.. W. S. M. Guerts van Kessel, and L. L. M. van Deenen. 1972. The properties of polyunsaturated lecithins in monolayers and liposomes and the interactions of these lecithins with cholesterol. *Biochim. Biophys. Acta.* **266:** 26–40.
43. Wessels, H., and N. S. Rajagopal. 1969. Die DC-Trennung von isomeren und naturlichen Triglycerid-Gemischen. *Fette Seifen Anstrichm.* **71:** 542–552.
44. Morris, L. J., D. M. Wharry, and E. W. Hammond. 1968. Chromatographic behaviour of isomeric long-chain aliphatic compounds. III. Thin-layer chromatography of positional isomers of substituted fatty acids and alcohols. *J. Chromatogr.* **33:** 471–479.
45. Gunstone, F. D., I. A. Ismail, and M. Lie Ken Jie. 1967. Thin layer and gas–liquid chromatographic properties of the *cis* and *trans* methyl octadecenoates and of some acetylenic esters. *Chem. Phys. Lipids.* **1:** 376–385.
46. Gunstone, F. D., and M. Lie Ken Jie. 1970. Thin layer and gas–liquid chromatographic properties of some *cis*-octadecenyl compounds. *Chem. Phys. Lipids.* **4:** 139–146.
47. Renkonen, O. 1966. Individual molecular species of phospholipids. III. Molecular species of ox-brain lecithins. *Biochim. Biophys. Acta.* **125:** 288–309.