POSITIONAL SPECIFICITY OF SATURATED AND UNSATURATED FATTY ACIDS IN PHOSPHATIDIC ACID FROM RAT LIVER

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SUMMARY

1. The relative incorporation of a number of radioactive fatty acids into the different glycerolipids of rat liver microsomes has been investigated.

2. Studies on the distribution of the radioactivity incorporated into phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid showed that in all three lipids, the majority of the saturated fatty acids were at the 1-position while the polyunsaturated fatty acids were largely confined to the 2-position.

3. The phosphatidic acid fraction of rat liver was isolated in a pure form, and its fatty acid distribution agreed with the results of the incorporation studies.

4. The incorporation of radioactive fatty acids into different molecular species of phosphatidic acid, separated as dimethyl phosphatidates was investigated.

It is now well established that the major glycerolipids in mammalian tissues possess distinct fatty acid patterns. Furthermore, the saturated or unsaturated fatty acids of any one glycerolipid are distributed between the two positions of the glycerol molecule in a nonrandom fashion. The important role of phosphatidic acid in the biosynthesis of both neutral glycerides and phospholipids[1,2] has suggested that the fatty acid profile incorporated into this lipid might be directly incorporated into its metabolic products. If phosphatidic acid were synthesized by specific enzymes which preferentially attached saturated fatty acids to the 1-position and unsaturated to the 2-position, the asymmetrical pattern of the fatty acids in the subsequent lipids might be explained.

Experiments were conducted investigating the relative incorporation of various radioactive fatty acids into the glycerophosphatides of rat liver microsomes. Observations on the incorporation of [14C]glycero-3-phosphoric acid into these lipids indicated that while phosphatidic acid might be synthesized *de novo*, most of the activity incorporated into phosphatidylcholine and the phosphatidylethanolamine was derived from transacylation reactions. The radioactive fatty acids incorporated into phosphatidic acid occupied specific positions in the molecule, saturated fatty acids being

found predominantly at the 1-position while unsaturated fatty acids were mainly confined to the 2-position. These observations differ from the results of LANDS AND HART[3,4] and STOFFEL, DE TOMAS AND SCHIEFER[5], but are consistent with the conclusions of HUSBANDS AND REISER[6]. Analysis of phosphatidic acid isolated from rat liver demonstrated that this lipid possesses an asymmetrical fatty acid distribution. The incorporation of various fatty acids into different species of phosphatidic acid, subfractionated on the basis of unsaturation could be rationalized in agreement with these conclusions.

Some of the data presented in this article have been reported previously[7,8].

EXPERIMENTAL

Incubation procedures

Microsomes, prepared as described previously[9] were incubated in 0.125 M KCl–0.02 M Tris buffer (pH 7.4) in the presence of 25 μmoles ATP, 0.3 μmole CoA (Fluka, Switzerland) under nitrogen at 37°. Other chemicals were added as indicated in the relevant tables. The final volume was 2.0 ml. Protein concentration was determined using the biuret method with 0.2 ml 5% sodium deoxycholate added[10]. The radioactive fatty acids (Radio-Chemical Centre, Amersham, England, or New England Nuclear, Boston, Mass., U.S.A.) were either dispersed in Tris–KCl by sonication for 1–2 min with an MSE 20-kcycle sonicator or complexed as their potassium salts to serum albumin[11].

The incubations were stopped by the addition of 6 ml of chloroform–methanol (1 : 2, v/v). The lipids were extracted according to the method of BLIGH AND DYER[12] and the upper phase was reextracted two times with 2 ml chloroform. The combined lipid extracts were evaporated to dryness under reduced pressure and the lipid residue was redissolved in a small volume of chloroform or chloroform–methanol (1 : 1, v/v). Aliquots of these extracts were then used to isolate the various lipids by means of thin-layer chromatography.

Reference substances

When the amount of a certain lipid in an incubation extract was too low to give sufficient staining, some unlabelled material was applied with the incubation sample to serve as an internal reference.

These marker lipids were obtained as follows: lecithin, isolated from egg yolk, and phosphatidylethanolamine extracted from rat liver, were purified by chromatography on silicic acid columns. The corresponding lyso derivatives were obtained by snake venom degradation. Phosphatidic acid was prepared by enzymic hydrolysis of pure egg lecithin or rat liver lecithin by a phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) preparation from Brussels sprouts in equal volumes of 0.1 M acetate buffer (pH 5.5) containing 0.01 M Ca^{2+} and ether. The phosphatidic acid was purified by column chromatography on silicic acid. Lysophosphatidic acid was prepared by phospholipase A hydrolysis of either the phosphatidic acid obtained from egg lecithin or synthetic 1-oleoyl-2-stearoyl-glycero-3-phosphate, which was a generous gift of Dr. P. P. M. BONSEN of this department. Phosphatidylinositol was kindly donated by Dr. A. D. SMITH (Oxford, England) and a sample of synthetic phosphatidylserine by Dr. G. H. DE HAAS of this department.

Resolution of lipid mixtures

Neutral lipids were separated on silica gel G thin-layer chromatographic plates using light petroleum–diethylether–formic acid (60:40:1,5, v/v/v), as a solvent. Lecithin and phosphatidylethanolamine were separated on silica gel G plates using chloroform–methanol–water (70:30:4, v/v/v) as a solvent. Phosphatidic acid was isolated on silica gel G plates prepared using 0.25 M oxalic acid instead of water. These plates could be developed with chloroform–methanol–hydrochloric acid (87:13:0.5, v/v/v[13]* However, during these studies it was found that with this solvent, the phosphatidic acid spot could be contaminated with monoacylglycerol*. Therefore phosphatidic acid was separated from the total lipid extract using light petroleum–acetone–formic acid (74:26:0.25, v/v/v)*. This system was more sensitive to overloading than the chloroform–methanol–hydrochloric acid system and cardiolipin tends to streak. However, under the conditions used, the lipid extracted from one incubation tube with 1 mg of phosphatidic acid added as an internal standard, could be separated on a single plate.

The plates were sprayed with a 0.005% solution of Rhodamine 6G and viewed under ultraviolet light. In cases in which only ^{14}C activity was present, the areas containing the relevant lipids were scraped and transferred directly to scintillation vials containing sufficient thixotropic gel powder (Cab-O-Sil, Packard) to prevent settling of the silica. Oxalic acid plates were neutralized for 10 min in NH_3 vapour. When the samples contained ^{3}H activity or when the lipids were to be treated further, the silica was transferred to a small column formed from a Pasteur pipette plugged with glass wool and the lipids were eluted with 50 ml of chloroform–methanol (lecithin (20:80, v/v); phosphatidic acid (1:1, v/v); dimethyl phosphatidates (80:20, v/v)). Samples eluted from silver nitrate plates were taken to dryness and washed according to the procedure of FOLCH, LEES AND SLOANE-STANLEY[14].

The determination of the positional distribution of the incorporated acid

Mixed dispersions of [3H]palmitate and [14C]linoleate, or [3H]stearate and [14C]linolenate or of [14C]linolenate and unlabelled stearate were prepared by sonicating 50 nmoles of both acids in Tris–KCl. The sonicates were incubated for 30 min with 10 mg of microsomal protein in the presence of 100 nmoles of glycero-3-phosphoric acid. The total lipid extract was separated on oxalic acid plates, the acidic plates were neutralized for 10 min in NH_3 vapour, and the silica containing the phosphatidic acid, phosphatidylcholine or phosphatidylethanolamine was scraped off after staining in iodine vapour. The lipids were extracted from the silica, using the two-phase system of BLIGH AND DYER with a 0.1 M borate buffer (pH 7.0) containing 5 mM Ca^{2+} as the aqueous component, in order to remove traces of oxalic acid possibly remaining behind in the chloroform layer and to adjust the pH. After evaporation of the bulk of the solvent, the chloroform extract was divided into two equal portions. 1 mg of egg lecithin was added to the phosphatidic acid sample, in order to promote enzyme action, the solvent was evaporated completely, and the lipid was dissolved in 5 ml of diethyl ether. To one tube was added 0.1 ml of Ca^{2+}-containing borate buffer with 2.5 mg of snake venom, and to the other one only buffer without enzyme, to serve as a control for nonenzymatic hydrolysis. The ether–buffer systems

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* Photographs are available from the authors by request.

were shaken vigorously for 3 h (phosphatidic acid) or 1 h (lecithin and phosphatidyl-ethanolamine) at room temperature. At the end of the incubation period a few ml of methanol were added to the mixture and it was dried under reduced pressure. The dried lipids were dissolved in a mixture of chloroform and methanol, and applied to regular plates for chromatography in chloroform–methanol–water (70:30:4, v/v/v), in the case of lecithin and phosphatidylethanolamine, or to oxalic acid plates for chromatography in chloroform–methanol–HCl (87:13:0.5, v/v/v), in the case of phosphatidic acid. Silicic acid containing lecithin, lysolecithin, phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidic acid, lysophosphatidic acid or free fatty acid was scraped from the plates, and the radioactivity was measured as described above. The distribution of radioactivity among the lyso derivative and the fatty acid fraction is indicative of the distribution of the incorporated fatty acid among the 1- and 2-position due to the positional specificity of the snake venom phospholipase. Phosphatidic acid degradation was not always complete, and since it has been reported[15] that several snake venoms catalyse hydrolysis of phospholipids at different rates depending on the type of fatty acid at the 2-position of the substrate, only experiments with more than 80% hydrolysis were used*. Furthermore, the results of experiments in which the no-enzyme control showed more than 10% hydrolysis were not taken into account.

Preparation and fractionation of dimethyl phosphatidates

    10 mg of phosphatidic acid prepared from rat liver lecithin by the action of phospholipase D was added as the free acid to the radioactive phosphatidic acid isolated from one-half of the incubation extracts. The combined phosphatidic acid was then converted to the free acid by washing according to the procedure of FOLCH, LEES AND SLOANE-STANLEY[14] using upper phase containing 0.1 M H_2SO_4 and then upper phase. The phosphatidic acid could then be quantitatively converted to its dimethyl derivative using 2 ml 0.2 M diazomethane in ether *plus* 0.1 ml methanol. The dimethyl phosphatidates were then separated into their different molecular classes on silicic acid plates impregnated with silver nitrate as described by REN-KONEN[16]. Equal aliquots of the dimethyl phosphatidates in chloroform were streaked onto two different plates. One plate (chloroform–methanol (98:2, v/v) was then used to separate the dimethyl phosphatidates containing 0, 1 and 2 double bonds. The other plate (chloroform–methanol–water 90:10:1, v/v/v) was used to separate the fractions containing 3, 4, 5 and 6 double bonds. The nature of the unsaturation of each fraction was determined by an analysis of its fatty acids *via* gas–liquid chromatography and comparison with the known molecular species of rat liver lecithin as determined previously[17]. The plates were sprayed with Rhodamine 6G and the lipids eluted as described above. All isolations and chromatography steps except the final elution were carried out using solvents containing butylated hydroxytoluene (50 mg/l), in order to avoid oxidation.

Degradation of dimethylphosphatidates with purified mold lipase

    Samples of radioactive dimethylphosphatidates from the incubations described

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* At the time that these experiments were performed, the pure pancreatic phospholipase A which exercises a better action on anionic phospholipids was not available.

above were degraded using a dispersion of purified mold lipase.* The dispersion was prepared by mixing 10 mg of sodium deoxycholate, 10 mg of rat liver lecithin, and 1.0 mg of purified lipase on a Vortex mixer with 10 ml of borate buffer (pH 6.5) containing 5 mM $CaCl_2$. After mixing, 0.05 ml of 30% serum albumin was added. The dimethyl phosphatidates were incubated with 2.0 ml of this dispersion for 15 min at 30°. After this incubation period, the hydrolysis was complete, as demonstrated by thin-layer chromatography using hexane–ether–acetic acid (20:80:1.0, v/v/v) as a developer. The lysodimethylphosphatidates and fatty acids produced were scraped from the plates and eluted with chloroform–methanol (50:50 and 80:20, v/v, respectively).

Radioactivity measurements

Radioactivity was measured in an automatic Packard Tricarb scintillation spectrometer. The scintillation mixture used was 16 ml of a solution of 80 mg 2,5-diphenyloxazole and 4.8 mg of 1,4-bis-2-(4-methyl-5-phenyoxazolyl)benzene in toluene. Quench corrections were made either by the external standard method or by the Channel-ratio procedure described by BRUNO AND CHRISTIAN[18].

Gas–liquid chromatography

Fatty acid analysis was carried out by gas–liquid chromatography as described previously[17].

Isolation of phosphatidic acid from rat liver

The total lipids from the livers of 30 female rats were extracted with 20 vol. of chloroform–methanol (2:1, v/v) and washed according to the method of FOLCH, LEES AND SLOANE-STANLEY[14] using upper phase containing 0.2% barium acetate. The chloroform layer was then taken to dryness, the residue was dissolved in 1 l of chloroform–methanol (2:1, v/v), and the phospholipids were converted to their barium salts by washing in the manner of FOLCH, LEES AND SLOANE-STANLEY[14] using upper phase containing 0.1 M H_2SO_4 (once) and 0.2% barium acetate (twice). The total lipids were then taken to dryness and applied to a column containing 500 g of activated, sieved silicic acid. The neutral lipids were completely eluted with chloroform. The column was then eluted with chloroform–methanol solutions in which the methanol concentration was raised by 0.5% increments until the eluate was phosphorus positive (1.5–4% methanol). The elution of phosphatidic acid was monitored using thin-layer chromatography on oxalic acid plates and chloroform–methanol–hydrochloric acid (87:13:0.5, v/v/v) and the phosphorous spray of DITTMER AND LESTER[19]. In this manner, the phosphatidic acid could be recovered relatively free from cardiolipin**.

The fractions containing phosphatidic acid were combined and further purified by thin-layer chromatography on oxalic acid plates using the chloroform–methanol–hydrochloric acid (87:13:0.5, v/v/v) system. The plates were sprayed with Rhodamine 6G and viewed under ultraviolet light. The areas containing phosphatidic acid were scraped and eluted with chloroform–methanol (1:1, v/v). Final purification was achieved by changing the phosphatidic acid into its sodium salt by passing through

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* This was a generous gift of Drs. P. LABOUREUR and M. LABROUSSE to Dr. G. H. DE HAAS.
** Photographs are available from the authors by request.

the resin Chelex 100 (Bio-Rad., Calif., U.S.A.), as described by CARTER AND WEBER[20], and chromatographing on columns of Sephadex LH-20 (Pharmacia, Uppsala, Sweden) with chloroform–methanol (2:1, v/v). The phosphatidic acid content was estimated to account for approx. 0.5% of the total liver phospholipids.

In order to minimize oxidation, butylated hydroxy-toluene (50 mg/l) was routinely added to all solvents except those for the Sephadex LH 20 chromatography. The spotting and developing of thin-layer chromatographic plates were carried out under a CO_2 atmosphere.

The product of this isolation was judged to be pure phosphatidic acid by the following criteria. The lipid gave a single spot corresponding to phosphatidic acid (prepared from either egg lecithin or rat liver lecithin with phospholipase D) on oxalic acid plates using the chloroform–methanol–HCl (87:13:0.5, v/v/v) and light petroleum–acetone–formic acid (74:26:0.25, v/v/v) systems.* On treatment of this material with purified phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) from pancreas, virtually complete degradation could be achieved[21]. The two products formed cochromatographed with fatty acid and 1-acyl-*sn*-glycero-3-phosphoric acid. Reaction with diazomethane yielded a product corresponding to dimethylphosphatidic acid that was derived from phosphatidic acid prepared from egg lecithin. Treatment of the fraction from rat liver with pancreatic lipase (EC 3.1.1.3)[21] produced compounds corresponding to fatty acid and 2-acyl dimethyl phosphatidate. It must be pointed out that this latter evidence is only confirmatory as other compounds also give rise to some dimethyl phosphatidate when treated with diazomethane[23,24]. After alkaline hydrolysis[25] of the isolated phosphatidic acid, only glycero-3-phosphoric acid could be detected on paper chromatography (phenol–saturated with water or propanol–ammonia–water (6:3:1, v/v/v)) or high-voltage electrophoresis. The glycero-3-phosphoric acid was located by staining with periodate/fuchsine–sulfurous acid[26].

Using the method described here, phosphatidic acid could be completely separated from cardiolipin The cardiolipins used were generous gifts from Dr. P. P. M. BONSEN (synthetic cardiolipin) and Dr. J. H. DE BRIJN (ox-heart cardiolipin). Furthermore, the fatty acid composition of the isolated phosphatidic acid contrasted sharply with that of cardiolipin obtained from the same lipid extracts (Table I), indicating that the phosphatidic acid isolated was not a breakdown product of cardiolipin.

TABLE I

THE FATTY ACID COMPOSITION OF RAT LIVER PHOSPHATIDIC ACID AND CARDIOLIPIN

Phosphatidic acid was isolated as described in the text except that Sephadex LH-20 chromatography was carried out on only 2 of the 5 samples. Cardiolipin was isolated from the total lipids by thin-layer chromatography with chloroform–methanol–water (65:35:4, v/v/v). Results are expressed as the percentage and standard deviations of analyses done on 5 groups of rats.

Fatty acid	Phosphatidic acid	Cardiolipin
16:0	32.8 ± 4.7	4.4 ± 1.9
16:1	+ +	2.6 ± 0.3
18:0	14.8 ± 5.2	1.6 ± 1.1
18:1	23.1 ± 4.2	14.9 ± 2.6
18:2	21.7 ± 1.5	77.5 ± 4.8
20:4	6.9 ± 4.6	

* Photographs are available from the authors by request.

RESULTS

Fig. 1 represents the ability of rat liver microsomal preparations to incorporate long-chain fatty acids into their lipids. All incubations were carried out in the presence of *rac*-glycero-3-phosphoric acid, since it was shown in preliminary studies that without this compound the incorporation of fatty acids into phosphatidic acid was usually very low. Under the conditions used, radioactive fatty acids were not incorporated into 3-*sn*-phosphatidylserine or 3-*sn*-phosphatidylinositol to an appreciable extent.
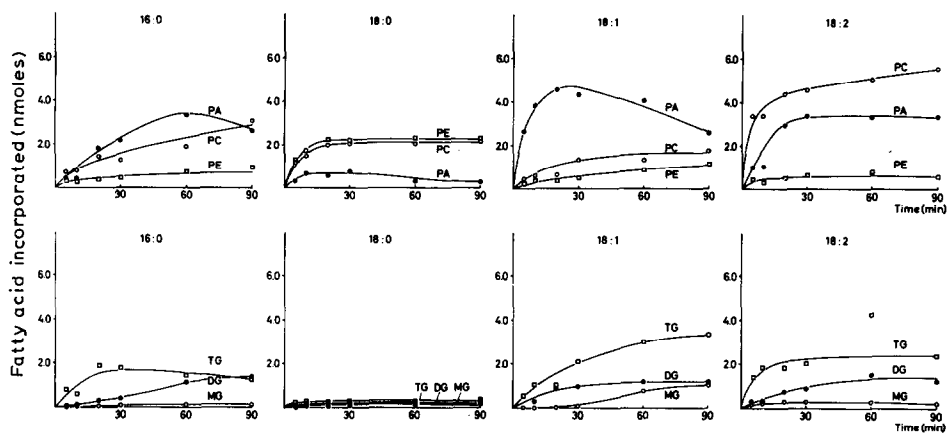

Fig. 1. The incorporation of palmitate, stearate, oleate and linoleate into various microsomal lipids. 20 nmoles of [^{14}C]fatty acid (serum albumin complex) were incubated for different time intervals with 10 mg rat liver microsomal protein. *rac*-Glycero-3-phosphoric acid (250 nmoles) and MgCl$_2$ (10 μmoles) were added. The incubations, extractions and isolations were carried out as described under METHODS. 16:0, palmitate; 18:0, stearate; 18:1, oleate; 18:2, linoleate. Abbreviations: PC, lecithin; PE, phosphatidylethanolamine; PA, phosphatidic acid; MG, monoacylglycerol; DG, diacylglycerol; TG, triacylglycerol.

Remarkable differences were observed in the incorporation patterns of different fatty acids. For example, phosphatidic acid incorporated palmitic and oleic acids more rapidly than did 3-*sn*-phosphatidylcholine or 3-*sn*-phosphatidyl ethanolamine. On the other hand, linoleic acid was rapidly incorporated into 3-*sn*-phosphatidylcholine and phosphatidic acid, but only slowly into 3-*sn*-phosphatidylethanolamine. In similar experiments it was observed that this pattern was also followed by linolenic acid[7]. Stearic acid was incorporated best into 3-*sn*-phosphatidylethanolamine and only slightly incorporated into phosphatidic acid.

The neutral lipid fraction was always labelled in the order: monoacylglycerols < diacylglycerols < triacylglycerols (Fig. 1). In general, it was observed that the incorporation into these lipids reflected the ability of the particular fatty acid to label phosphatidic acid. This relationship was more clearly demonstrated in some experiments in which the incorporation into phosphatidic acid was somewhat lower than normal.

Data on the incorporation of arachidonic acid are lacking in Fig. 1 due to the limited availability of this acid. However, the data from a few fixed-time experiments

TABLE II

25 nmoles of [³H]arachidonic acid (sonicated) were incubated under regular conditions with 5 mg microsomal protein for 30 min. After extraction and isolation, the distribution of the radioactivity among the lipids was determined and calculated as a percentage of the radioactivity recovered.

Expt. No.	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidic acid	Diacylglycerol + triacylglycerol	Free fatty acid
1	53.7	12.5	7.3	8.6	9.0
2	68.3	14.9	4.3	3.7	7.0
3	65.2	11.4	11.4	4.5	6.5

with tritiated arachidonic acid* indicated that it is preferentially incorporated into lecithin (Table II).

As previously mentioned, very little incorporation into phosphatidic acid was observed unless glycero-3-phosphoric acid was present. Fig. 2 represents an investigation into the role of glycero-3-phosphoric acid as a lipid precursor. While all lipids were labelled, it can be seen that most of the activity was limited to phosphatidic acid. Diacylglycerol was also substantially labelled, presumably through the action of phosphatidate phosphohydrolase (EC 3.1.3.4).
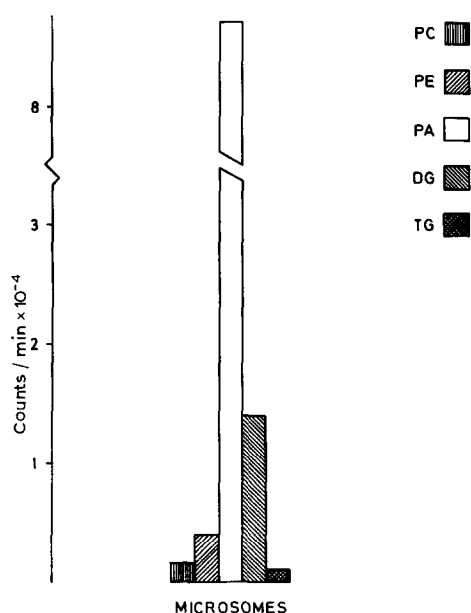

Fig. 2. The incorporation of [¹⁴C]glycerol-3-phosphoric acid into rat liver microsomes. 5 mg of microsomal protein were incubated for 30 min under standard conditions with $4 \cdot 10^5$ counts/min of [¹⁴C]glycerol-3-phosphoric acid. The phospholipids were separated on oxalic acid plates using chloroform–methanol–hydrochloric acid (87:13:0.5, v/v/v). Diacylglycerol and triacylglycerol were separated on normal plates using light petroleum–ether–formic acid (60:40:1.5, v/v/v). Abbreviations: see legend to Fig. 1.

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* The authors are indebted to Dr. D. A. VAN DORP and Dr. A. K. BEERTHUIS (Unilever, Vlaardingen) for a sample of [5,6,8,9,11,12,14,15-³H₃]arachidonic acid.

The low incorporation of glycero-3-phosphoric acid into the remaining lipids implies that *de novo* synthesis is not responsible for the fatty acid incorporations into these lipids. Presumably, this incorporation takes place mainly through the acylation of endogenous 1- and 2- acyl lysophosphatidates [27-29] formed by phospholipase A_1 and A_2 (ref. 30), and the acylation of diacylglycerol to form triacylglycerol[31].

Table III represents the results of a number of investigations on the positional

TABLE III

POSITIONAL DISTRIBUTION OF RADIOACTIVE FATTY ACIDS INCORPORATED INTO LECITHIN, PHOS-PHATIDYLETHANOLAMINE AND PHOSPHATIDIC ACID

50 nmoles of [³H]palmitic, [¹⁴C]linoleic, [³H]stearic or [¹⁴C]linoleate were incubated with 10 mg of microsomal protein in the presence of 100 nmoles glycerophosphate. After isolation of thin-layer chromatography, the lipids under investigation were degraded by phospholipase A from snake venom and the distribution of radioactivity among the free fatty acid and the lyso derivative determined. Mean values of 2–6 experiments are presented.

Fatty acid	Lecithin		Phosphatidyl ethanolamine		Phosphatidic acid	
	1-position [%]	2-position (%)	1-position (%)	2-position (%)	1-position (%)	2-position (%)
16:0	83	17	87	13	72	28
18:0	90	10	86	14	86	14
18:2	6	94	22	78	16	84
18:3	12	88	32	68	20	80

distribution of the fatty acids incorporated into phosphatidic acid in rat liver microsomes. It can clearly be seen that the 1-hydroxyl group of the added glycero-3-phosphoric acid is esterified mainly by palmitic or stearic acid, whereas linoleic and linolenic acid are esterified predominantly at the 2-position. In fact, the asymmetry of the phosphatidic acid molecule is almost as pronounced as that in lecithin. Similar experiments demonstrated that the phosphatidic acid molecules synthesized by rat liver mitochondria[7,8] and rat liver slices (unpublished results) possessed a similar asymmetry.

In view of the obvious disparity between the results in Table III and those

TABLE IV

COMPOSITION AND DISTRIBUTION OF THE FATTY ACIDS FROM RAT LIVER PHOSPHATIDIC ACID

Phosphatidic acid was isolated from rat liver as desribed in the methods and hydrolysed with pancreatic phospholipase A. The degradation products were isolated and analysed by gas–liquid chromatography on an F & M gas chromatograph model 720.

Sample	Fatty acid		Total	1-position	2-position
I	Palmitic	16:0	28.1	51.2	8.2
	Palmitoleic	16:1	2.6	1.0	3.3
	Stearic	18:0	17.0	32.7	2.7
	Oleic	18:1	19.3	7.4	29.7
	Linoleic	18:2	21.9	4.5	35.3
	Arachidonic	20:4	11.1	2.1	20.3
II	Palmitic	16:0	27.2	45.3	7.6
	Palmitoleic	16:1	++	++	2.3
	Stearic	18:0	22.8	38.2	2.6
	Oleic	18:1	18.8	10.9	30.0
	Linoleic	18:2	21.5	5.6	34.3
	Arachidonic	20:4	9.6	0.0	24.9

reported by LANDS AND HART[3,4] and STOFFEL, DE TOMAS AND SCHIEFER[5], it was decided to reinvestigate this matter using a different approach, *i.e.*, the examination of the fatty acid distribution of the endogenous phosphatidic acid. The fatty acid pattern of phosphatidic acid from rat liver and its distribution among the 1- and 2-positions is given Table in IV. The phosphatidic acid was hydrolysed by means of purified pancreatic phospholipase A, as described by DE HAAS, BONSEN AND VAN DEENEN[13]. The products were separated on oxalic acid plates and the fatty acid composition examined by gas–liquid chromatography. At least 90% degradation was obtained in these hydrolyses.

Examination of the total fatty acids of rat liver phosphatidic acid (see also Table I) revealed that approx. 50% of the fatty acids are saturated. The 1-acyl-lysophosphatidic acid contained more than 80% saturated acids while the fatty acids released from the 2-position are almost 90% unsaturated. Even oleic acid, which shows the lowest specificity, favoured the 2-position by a factor of at least 3:1.

The fatty acid composition of phosphatidic acid agrees rather well with that which one would envisage from the acylation studies presented in Fig. 1 and Table II, with the possible exception that the stearic acid content is somewhat too high.

HÜBSCHER AND CLARK[32] have reported the isolation of phosphatidic acid from rat, ox and pig liver. The fatty acid pattern they observed was similar to the one observed for cardiolipin in the present studies (Table I). While these workers presented excellent analytical data concerning their product, the fact that they did not use thin-layer chromatography techniques makes it somewhat difficult to interpret their results. GRAY[33] has reported the isolation of phosphatidic acid from Landschutz ascites tumour. He observed that the fatty acid composition was similar to that of 3-*sn*-phosphatidylethanolamine. Using methods similar to those used in the present report, SHELTAWY AND DAWSON[34] isolated phosphatidic acid from the myelin fraction of rats injected with $^{32}P_i$. They were able to demonstrate that the isolated phosphatidic acid had the same specific activity as the glycerol-3-phosphoric acid produced by alkaline hydrolysis of myelin lipids.

In view of this evidence for the asymmetrical nature of the phosphatidic acid molecule in rat liver microsomes, it was decided to investigate the nature of the incorporation of various fatty acids into the different molecular species of this lipid. The radioactive fatty acids were incubated in pairs with rat liver microsomes as indicated in Table V. The phosphatidic acid was isolated and converted to dimethyl phosphatidates as described in the methods section and chromatographed on plates impregnated with silver nitrate. When palmitic acid was incubated in the presence of oleic acid, there was a limited incorporation into the species containing no double bonds. That small quantities of disaturated molecules only were produced is not surprising in view of the small amount of palmitic or stearic acid found at the 2-position of rat liver phosphatidic acid (Table IV). The most significant incorporation of palmitic acid in the presence of oleic acid was into the $\Delta 1$ spot. This indicates that there was a production of the species containing palmitic and oleic acid mainly as acyl constituents. This view is corroborated by the high incorporation of oleic acid into the $\Delta 1$ fraction. Palmitic acid was also incorporated to some extent into the $\Delta 2$ fraction, presumably giving rise to the species 1- palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoric acid. The good incorporation of oleate into the $\Delta 2$ fraction is probably due to the formation of dioleoyl-*sn*-glycero-3-phosphoric acid.This agrees with the observation that oleic

TABLE V

THE INCORPORATION OF RADIOACTIVE FATTY ACIDS INTO VARIOUS MOLECULAR SPECIES OF PHOS-
PHATIDIC ACID IN RAT LIVER MICROSOMES

20 nmoles of each fatty acid (serum albumin complex) were incubated with 10 mg microsomal
protein in the presence of 250 nmoles *rac*-glycero-3-phosphoric acid and 10 μmoles MgCl$_2$. Each
incubation flask contained 2 fatty acids labelled with ^{3}H or ^{14}C as indicated. Phosphatidic acid
was isolated, converted to its dimethyl derivative and separated on AgNO$_3$ plates as described in
the text. Results are expressed as nmoles incorporated.

Fatty acid Expt. I	Double bonds	^{3}H		^{14}C	
		15 min	*90 min*	*15 min*	*90 min*
^{3}H-16:0	0	0.02	0.18	0.01	0.13
^{14}C-18:1	1	0.19	0.70	0.67	2.69
	2	0.10	0.44	1.22	1.24
	3	0.03	0.16	0.10	0.54
	4	0.04	0.12	0.16	0.52
	5	0.00	0.03	0.01	0.06
	6	0.00	0.08	0.02	0.16
^{3}H-16:0	0	0.03	0.07	0.06	0.03
^{14}C-18:2	1	0.13	0.23	0.10	0.20
	2	0.15	0.42	0.42	1.16
	3	0.04	0.10	0.16	0.31
	4	0.03	0.12	0.22	0.70
	5	0.00	0.05	0.02	0.14
	6	0.01	0.05	0.01	0.09
^{3}H-18:0	0	0.03	0.04	0.05	0.04
^{14}C-18:1	1	0.10	0.28	0.72	1.37
	2	0.05	0.47	0.97	2.21
	3	0.02	0.10	0.07	0.50
	4	0.03	0.16	0.21	0.42
	5	0.00	0.03	0.02	0.05
	6	0.00	0.05	0.02	0.12
^{3}H-18:0	0	0.03	0.05	0.06	0.04
^{14}C-18:2	1	0.05	0.21	0.13	0.31
	2		0.54		1.38
	3	0.02	0.15	0.21	0.61
	4	0.04	0.09	0.18	0.58
	5	0.00	0.01	0.03	0.10
	6	0.03	0.05	0.04	0.17
Expt. II					
^{3}H-16:0	0	0.44	0.50	0.01	0.03
^{14}C-18:1	1	1.19	4.95	3.04	2.50
	2	0.31	0.50	1.63	1.83
	3	0.02	0.03	0.12	0.15
	4	0.04	0.03	0.07	0.05
	5	0.05	0.02	0.03	0.02
	6	0.06	0.14	0.04	0.04
^{3}H-16:0	0	0.74	0.62	0.02	0.01
^{14}C-18:2	1	0.98	0.00	0.15	0.00
	2	1.02	0.95	1.98	2.26
	3	0.08	0.02	0.06	0.04
	4	0.07	0.03	0.20	0.07
	5	0.05	0.02	0.03	0.02
	6	0.11	0.08	0.03	0.02
^{3}H-18:0	0	0.08	0.10	0.02	0.03
^{14}C-18:1	1	0.39	0.40	3.73	2.76
	2	0.17	0.20	1.55	1.64
	3	0.01	0.01	0.16	0.11
	4	0.05	0.05	0.08	0.07
	5	0.00	0.01	0.02	0.03
	6	0.04	0.04	0.06	0.05
^{3}H-18:0	0	0.18	0.12	0.03	0.18
^{14}C-18:2	1	0.34	0.25	0.40	0.07
	2	0.51	0.64	3.04	2.50
	3	0.01	0.04	0.06	0.15
	4	0.02	0.08	0.14	0.23
	5	—	0.07	—	0.07
	6	0.05	0.05	0.05	0.04

acid exhibited the least asymmetric distribution of the fatty acids found in the endogenous rat liver phosphatidic acid. In the presence of linoleic acid, the best incorporation of palmitate was found to take place in the Δ2 fraction. Although in Expt. I the incorporation of palmitic acid into the Δ2 spot was the same as that in the presence of oleic acid, normally the addition of linoleic acid stimulated the uptake of palmitate into the Δ2 spot. Because linoleic acid was also predominantly incorporated into this fraction, it is feasible that the species 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoric acid was formed. Incorporation of linoleate into the Δ4 fraction may be due to the production of a dilinoleoyl species. When stearic acid was incubated in the presence of oleic acid, there was no significant uptake into the fraction containing disaturated molecules. A small, though substantial incorporation of stearate was observed into the Δ1 fraction, which indicates that the species 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoric acid was being formed. The rather high incorporation of stearate into the Δ2 spot could be explained by the formation of 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphorylcholine due to a high endogenous level of linoleate. A good incorporation of oleic acid into the Δ1 and the Δ2 fraction was observed, indicating that apart from 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine, a high production of dioleoyl-*sn*-glycero-3-phosphoric acid occurred. As mentioned above, significant amounts of the latter species were also formed in the presence of palmitic acid. As with palmitic acid, oleic acid is also incorporated into the Δ3 and Δ4 fractions. In the presence of stearate, linoleate was incorporated mainly into the Δ2 fraction so as to give 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoric acid. As in the experiments in which palmitic acid was added as the saturated component, a notable uptake of linoleic acid could be observed into the Δ3 and Δ4 fractions.

It is interesting to note that palmitic and stearic acids are not incorporated into the more highly unsaturated areas. This is particularly interesting in view of the observation that 1-stearoyl-2-arachidonoyl-3-*sn*-phosphatidylcholine is a major species in normal rat liver. This result indicates that the more highly unsaturated fatty acids are introduced through the reacylation of the individual phosphatides. The studies of HILL AND LANDS[35] on the relative rates of acylation of 1-acyl-glycero-3-phosphoric acid and 1-acyl-glycero-3-phosphorylcholine support such a view, but do not explain why rat liver contains more 1-stearoyl-2-arachidonoyl-3-*sn*-phosphatidylcholine than 1-palmitoyl-2-arachidonoyl-3-*sn*-phosphatidylcholine. In order to be able to interpret the data in Table V more exactly and support the tentative conclusions about the species formed, some of the radioactive samples were degraded with a purified lipase from mold in order to establish the positional distribution of the incorporated fatty acids. This enzyme is known to release specifically the fatty acids from the 1-position of dimethyl phosphatidates[36]. The hydrolysis was carried out until completion. The results compiled in Table VI, in reasonable agreement with the degradation of the parent phosphatidic acid molecules by phospholipase A, demonstrate a nonrandom distribution of the incorporated fatty acids, except for oleic acid. This component exhibited a rather random distribution. Palmitic, and even more markedly, stearic acid are directed preferentially to the 1-position, this being valid not only in the total dimethyl phosphatides but also in the monoenoic and dienoic species. As one would expect, linoleic acid incorporated preferentially into the 2-position, though the asymmetric distribution of this acid is less pronounced than in phosphatidylcholine.

TABLE VI

POSITIONAL DISTRIBUTION OF THE RADIOACTIVE FATTY ACIDS IN SOME SPECIES OF DIMETHYL PHOS-
PHATIDATES DERIVED FROM RAT LIVER MICROSOMAL PHOSPHATIDIC ACID

Some of the samples indicated in Table V were incubated with purified mold lipase as described under METHODS. The degradation products were separated on silica thin-layer plates using hexane–ether–acetic acid (20:80:1, v/v/v) as a developer, and the distribution of the radioactivity was determined.

Samples	Fatty acid	1-position (%)	2-position (%)
Total dimethylphosphatides	16:0	74	26
	18:2	32	68
	18:0	92	8
	18:1	48	52
Monoenoic dimethylphosphatides	16:0	73	27
	18:1	48	52
	18:0	*	*
	18:1	47	53
Dienoic dimethylphosphatides	16:0	96	4
	18:2	31	69
	18:0	*	*
	18:2	34	66

* Insufficient radioactivity for satisfactory comparison.

DISCUSSION

LANDS AND HART[3] have suggested that the asymmetric distribution of fatty acids in the glycerophosphatides may be brought about by the combined action of phospholipases and selective acylating enzymes on phospholipids initially formed by *de novo* synthesis and having a random fatty acid distribution. The random acylation of glycero-3-phosphoric acid, a prerequisite for this view, was indeed observed by these workers in guinea pig liver microsomes. In a later study[4], these authors recognized that this reaction might be more specific in other biological systems but still concluded that the acylation of the 1-position of glycero-phosphate, at least, was nonspecific. STOFFEL, DE TOMAS AND SCHIEFER[5] observed only small differences in the acylation rates of saturated and unsaturated fatty acids to 1-palmitoyl-glycero-3-phosphoric acid.

The observations presented in this article, which confirm those reported by HUSBANDS AND REISER[6], lead to a different conclusion. Saturated fatty acids incorporated into phosphatidic acid were found mainly at the 1-position, while unsaturated fatty acids were chiefly confined to the 2-position. Thus, the metabolic products of phosphatidic acid could already possess an asymmetry with respect to saturated and unsaturated fatty acids, without the intervention of lyso-acylating enzymes. In a recent abstract, HILL AND LANDS[37] drew a similar conclusion from their experiments with rat liver slices. This indicates that the selective acylation of lysophosphatides, in combination with phospholipase activity, maintains rather than introduces the asymmetric distribution of fatty acids[8]. On the other hand, this mechanism probably plays an inportant role in producing more subtle changes in the fatty acid pattern. It is considered likely that the deacylation–reacylation cycle plays a role in the introduction of the polyunsaturated acids to the 2-position. However, the contributions

of other pathways such as the phosphorylation of mono- and diacylglycerol[38] or the methylation of one nitrogenous base to form another[39] must not be overlooked.

The mechanism by which the asymmetric distribution of fatty acids in phosphatidic acid is effected is not yet elucidated. At least two alternative explanations might be given: either the enzymes acylating *sn*-glycero-3-phosphoric acid are positionally specific, or the initially randomly incorporated fatty acids are redistributed by the action of phospholipases and selective acyl-CoA:monoacyl-glycero-3-phosphate acyltranferases. If the former explanation is true, it would mean that the enzyme which first acylates glycero-3-phosphoric acid must be rather specific for saturated and unsaturated fatty acids as well as being able to carry out the crucial primary step of lipid synthesis. It seems somewhat unlikely that the properties of this critical enzyme in guinea pig liver microsomes would be so different from that in rat liver microsomes. On the other hand, the relative rates of the readjustment (Mechanism 2) might well differ between rat and guinea pig livers.

If the latter mechanism were operative, we might expect to find a somewhat random incorporation of fatty acids into the molecular species of phosphatidic acid at early stages in the incubation. This random incorporation might be expressed as unexpectedly high labelling of di-saturated or di-unsaturated species. At later stages in the incorporation, the activity of these "unnatural" species would decline as the fatty acids were rearranged. The data in Table V do not support this conclusion. However, as the reorganization of the fatty acids in rat liver phosphatidic acid may take place at a rate comparable to or even faster than the original acylation, no definite conclusion can be reached. Also, one cannot exclude the theoretical possibility that the first step in the acylation of glycero-3-phosphoric acid is random. This would mean that both saturated and unsaturated 1-acyl- and 2-acyl-*sn*-glycero-3-phosphoric acid are formed. From these products the saturated 1-acyl- and the unsaturated 2-acyl isomer could be chosen selectively for the selective incorporation of an unsaturated and saturated fatty acid, respectively, resulting in an asymmetric distribution of the acyl chains in the end product, phosphatidic acid.

A great deal remains to be learned concerning the enzyme which initially acylates *sn*-glycero-3-phosphoric acid. It is not known whether this enzyme acylates the 1- or 2-position or both. LANDS AND HART[4] favour a 1-position acylation. These authors have clearly demonstrated that a different enzyme can be involved in the more rapid acylation of the monoacylglycero-3-phosphoric acid.

While it has not been possible to obtain enzyme(s) capable of only the initial acylation in mammalian tissues[40], some success has been reported in bacterial systems[41,42].

Until lately, it appeared that the enzyme catalysing the first acylation of *sn*-glycero-3-phosphoric acid played a unique role in that it catalysed the only known *de novo* formation of glycerolipids. In recent notes, RAO, SORRELS AND REISER[43,44] and HAJRA AND AGRANOFF[45] have reported that *rac*-glyceraldehyde-3-phosphoric acid and dihydroxyacetone phosphate can also be acylated by liver enzymes. The enzymes acylating these tissue phosphate esters appear to differ from the glycero-3-phosphate acylating enzyme. RAO, SORRELS AND REISER suggest that different specificities in acylating the primary hydroxyl group of dihydroxyacetone phosphate and the secondary hydroxyl group in glyceraldehyde-3-phosphoric acid may be responsible for the acyl distribution in glycerides. These results demonstrate that the initial pro-

cesses concerned with glycerolipid biosynthesis are more complex than previously realized and that further research is necessary before definite conclusions can be reached.

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