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The Fractionation of Phosphatidylinositol into Molecular Species by Thin-Layer Chromatography on Silver Nitrate-Impregnated Silica Gel

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1. Two methods for the fractionation of phosphatidylinositol into molecular species were developed. In addition to preserving the fatty acid moiety of the molecule, the first method preserves the phosphorus, and the second preserves both the phosphorus and inositol ring. 2. In the first method, phosphatidylinositol was oxidized with periodate and the products reacted with diazomethane. I.r. examination showed that the main product was identical with dimethylphosphatidic acid. Fractionation to molecular species was carried out on thin layers impregnated with silver nitrate. The fatty acid composition of the species was determined by gas–liquid chromatography, and their distribution in lamb liver phosphatidylinositol was studied by a method using [^{3}H]methanol. 3. In the second method, phosphatidylinositol was acetylated under mild reaction conditions. The major product was the triacetylated derivative of this phospholipid. This was reacted with diazomethane and the methylated–triacetylated phosphatidylinositol was fractionated into molecular species on silver nitrate-impregnated thin layers. Solvent mixtures containing acetone and distilled chloroform were found most suitable for this purpose. The fatty acid composition of the molecular species was determined by g.l.c., and their distribution in lamb liver phosphatidylinositol was studied by a method using [1-^{14}C]acetic anhydride during the acetylation reaction. 4. Results from both methods agree fairly well. The most predominant species of lamb liver phosphatidylinositol is the monoenoic (60%) followed by the tetraenoic (17%). The di- and tri-enoic species existed as minor components.

The metabolic heterogeneity of molecular species of rat liver phosphatidylcholines was demonstrated almost 12 years ago (Collins, 1960; Harris & Robinson, 1960). This was later confirmed and it was further demonstrated that some enzymes involved in the metabolism of phospholipids are specific to their fatty acid composition (Kanoh, 1969). Considerable work in several laboratories (Arvidson, 1965; Renkonen, 1967; also reviewed by Viswanathan, 1969) was therefore directed towards developing reliable methods for the analysis of phospholipid classes into molecular species. All these studies were conducted on phosphatidylcholine and phosphatidylethanolamine mainly due to their availability in large amounts. Hence a great need existed for developing similar techniques to fractionate minor phospholipids into molecular species.

The techniques so far developed for major phospholipids can be divided into two types. In the first, the fractionation of either the intact or masked phospholipid is by direct t.l.c. on $AgNO_3$-impregnated silica gel H; in the second this chromatographic step is performed after the chemical or enzymic hydrolysis of the phospholipids into diglycerides. Methods developed by Renkonen (1968) to mask the phosphate group (first type) involve the conversion of a phospholipid into dimethylphosphatidic acids. The obvious advantage of these types of methods is that they enable a study to be made of the turnover of both the polar (phosphorus and base) and non-polar (fatty acids) parts of the molecule.

We are interested in this laboratory in the metabolism of phosphoinositides in the nervous system and we selected therefore to develop methods for the analysis of molecular species of phosphatidylinositol. None of the above methods of masking the phosphate group were found applicable to phosphatidylinositol. Under the various conditions attempted, this phospholipid can only be attacked to a small extent by the soluble phospholipase D from savoy cabbage. Similarly, little conversion into dimethylphosphatidic acid occurs on direct interaction with diazomethane.

We report in this paper two methods simultaneously developed to fractionate phosphatidylinositol into different molecular species. The first involved the oxidation of the inositol ring with periodate and reacting the products with diazomethane to produce dimethylphosphatidic acid. The second involved masking the inositol ring by acetylation and the phosphate by methylation. This product

and dimethylphosphatidic acid were separately fractionated to molecular species by t.l.c. on $AgNO_3$-impregnated silica gel G.

Experimental and Results

General methods

Preparation of phosphatidylinositol. The phosphatidylinositol used for these studies was extracted either from lamb liver or ox brain and purified by the method of Luthra & Sheltawy (1972).

Thin-layer chromatography. All chromatographic separations were carried out at 4°C. An 'S-Chamber' (Sheltawy & Dawson, 1969) was used with t.l.c. on silica gel H.

Reaction conditions. The final conditions of reactions employed are given below, and were followed in the order described for each method.

Method 1. *Fractionation of molecular species of phosphatidylinositol after conversion into dimethylphosphatidic acid*

Periodate oxidation. A sample of phosphatidylinositol (2.82mg of P) was dissolved in 3.7ml of chloroform–ethanol (2:5, v/v) and 1.1ml of water was added. Sodium periodate (108mg/8ml of chloroform–ethanol–water; 2:5:2, by vol.) was then added and the reaction carried out in the dark under N_2 for 17h at room temperature. The reaction was stopped by adding 1ml of glycerol to consume the unchanged periodate. Chloroform (11.3ml) and 0.9% NaCl (9.6ml) were added and the lipid products recovered in the chloroform phase were further purified by washing with 7.0ml of ethanol–1M-HCl (2:3, v/v).

Negligible loss of esterified fatty acids (Augustyn & Elliott, 1969) was found under these conditions. Four major bands were obtained when the lipid

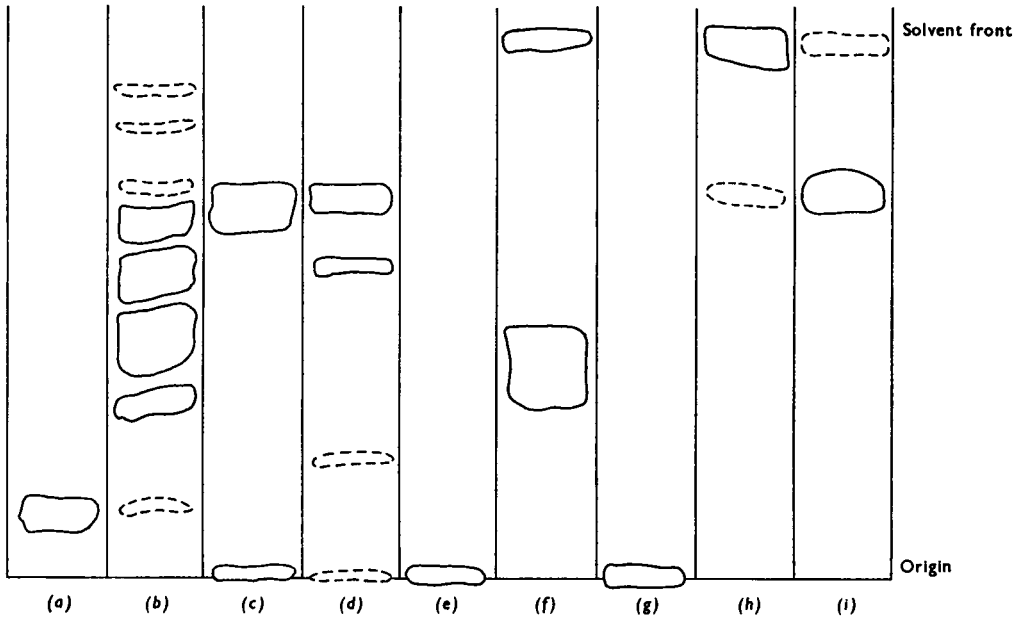

Fig. 1. *T.l.c. of different reaction products*

All batches of silica gel H used were previously washed with organic solvents as described by Sheltawy & Dawson (1969). The layers (0.25mm thick) were made of silica gel G in (*c*), (*d*), (*g*), (*h*) and (*i*), and of silica gel H impregnated with 1% potassium oxalate in (*a*), (*b*), (*e*) and (*f*). Developing solvents were chloroform–methanol–acetic acid–water (65:24:5:1, by vol.) in (*a*) and (*b*), ether in (*c*), (*d*), (*g*), (*h*) and (*i*), and chloroform–methanol (7:3, v/v) in (*e*) and (*f*). Samples in the different lanes were: (*a*) and (*e*) phosphatidylinositol from lamb liver; (*b*) its periodate oxidation products; (*d*) the products of their diazomethanolysis; (*c*) standard dimethylphosphatidic acid produced from egg phosphatidylcholine; (*f*) acetylation products of lamb liver phosphatidylinositol; (*g*) the triacetylated derivative; (*h*) and (*i*) the products of its methylation. Broken lines indicate minor components.

reaction products were subjected to t.l.c. on layers of washed silica gel H impregnated with potassium oxalate (Gonzalez-Sastre & Folch-Pi, 1968), with chloroform–methanol–acetic acid–water (65:25:5:1, by vol.) as solvent. These bands presumably represented the cleavage of the inositol ring at different positions (Figs. 1*a* and 1*b*).

Diazomethanolysis. The periodate oxidation products in 1.5ml of chloroform were treated with 16ml of ethereal 0.16M-diazomethane in a stoppered glass tube. The mixture was incubated in the dark at 29°C for 14h, and the reaction terminated by passing a current of N_2 over the surface to drive off the unchanged reagent.

When the lipid products of diazomethanolysis were chromatographed on thin layers of silica gel G (washed with organic solvents) with ether as the developing solvent, three spots were obtained, plus unchanged material at the origin (Fig. 1*d*). The major one (73% of the original phosphatidylinositol P) corresponded to dimethylphosphatidic acid standard (Fig. 1*c*). The remaining two spots presumably represented monomethylphosphatidic acid and the diazomethanolysis products of minor lipids from the oxidation reaction. Preparative t.l.c. of the major spot gave rise to a pure sample of dimethylphosphatidic acid. The i.r. spectrum of this is shown in Fig. 2(*c*) compared with standard dimethylphosphatidic acid (Fig. 2*b*) and the original sample of phosphatidylinositol (Fig. 2*a*). The main changes due to methylation of the phosphate group can be summarized as follows: (i) a considerable decrease in the intensity of absorption at 3300cm^{-1} due to hydrogen-bonded OH; (ii) an increase in intensity of absorption at 1280, 1170 and 1040cm^{-1} due to the C–O–P bonds; (iii) a decrease in the intensity of peaks at 1000cm^{-1}

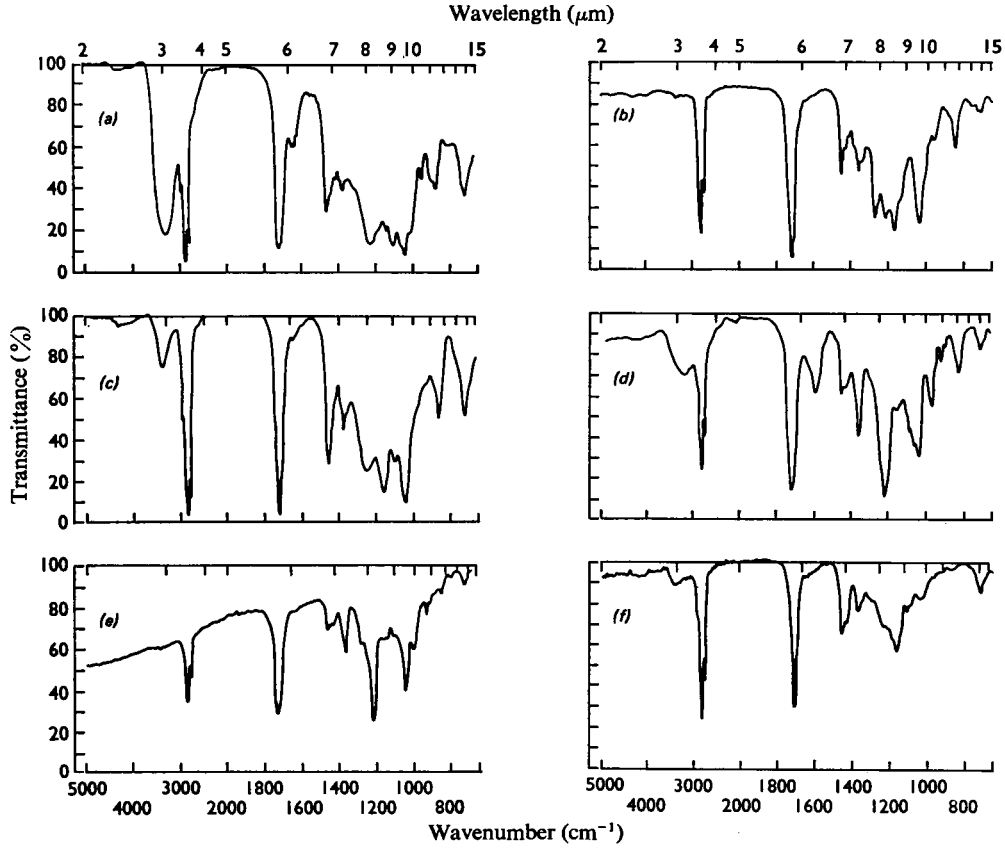

Fig. 2. *Infrared spectra of lamb liver phosphatidylinositol and derivatives*

(*a*) Phosphatidylinositol, (*b*) dimethylphosphatidic acid from egg phosphatidylcholine, (*c*) dimethylphosphatidic acid from periodate oxidation products of lamb liver phosphatidylinositol, (*d*) triacetylphosphatidylinositol, (*e*) and (*f*) methylation products of triacetylphosphatidylinositol.

due to the inositol ring. These changes are similar to those described by Crone (1964).

The dimethylphosphatidic acid produced had a similar fatty acid composition to that of the original phosphatidylinositol (Table 1).

T.l.c. on $AgNO_3$-impregnated silica gel G. $AgNO_3$ (6g) was mixed with 20g of silica gel G slurry. The layers (0.5mm thick) were activated at 110°C for 1h and used immediately, with methanol–chloroform (1:49, v/v) as solvent.

Initial attempts at fractionation of dimethylphosphatidic acid revealed that although a separation of molecular species was achieved, the pattern was confused by the presence of fatty acid impurities. Examination of the organic solvents used, the localization dye, the adsorbent and particularly the $AgNO_3$ revealed that all these chemicals contained impurities that corresponded on g.l.c. to the methyl esters of $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{17:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ fatty acids. Prior purification of all the reagents and the use of suitable blanks extracted from the plates after development enabled us to eliminate interference from any impurities.

Table 1 shows the fatty acid composition of the separated bands obtained from preparative t.l.c. Seven bands including the origin were obtained (Fig. 3*a*). Each band contained a single molecular species except band 1 and the origin, which represented an incomplete fractionation of the penta- and hexa-enoic species respectively. A certain degree of autoxidation in the tri- and tetra-enoic bands was noticeable. The pattern in the dienoic band is masked due to the possible presence of nonadecanoic acid ($C_{19:0}$), which has a retention time almost identical with that of linoleic acid on the polyester columns used (see Table 1 for conditions used for g.l.c.).

Distribution of molecular species in phosphatidylinositol from lamb liver. The bands together with a representative blank area were scraped off the plate and eluted with a suitable volume of chloroform–methanol (3:1, v/v). The lipids were separated from the marker dye (dichlorofluorescein) and $AgNO_3$ by distribution between the two phases of chloroform–methanol–0.9% NaCl (4:2:3, by vol.). The solvent was removed from the lower phase and the lipids were dissolved in 0.14ml of chloroform–[^{3}H]methanol–BF_3 in ether (sp.gr. 1.13) (25:75:4, by vol.). The reaction was conducted at 37°C for 2h and stopped by a stream of N_2. To limit contamination with unchanged [^{3}H]methanol, the methyl esters were distributed in 3.2ml of methanol–water–light petroleum (b.p. 40–60°C) (2:15:15, by vol.). Portions of the upper phase were evaporated under reduced pressure and dissolved in 10ml of scintillant (0.5% 2,5-diphenyloxazole in toluene). The distribution of radioactivity in the different fractions was taken as a measure of the [^{3}H]methyl esters therein.

Direct methods of chemical determination of the

Table 1. *Fatty acid distribution in different molecular species of lamb liver phosphatidylinositol fractionated by method 1*

Results are expressed as mol/100mol of total fatty acids recovered. Bands are numbered from the origin (0) of the $AgNO_3$-impregnated thin layer to the solvent front (e.g. band no. 6, saturated). A 6% (w/w) polyethylene glycol adipate column on Phasesep NI (DCMS, 100–120 mesh) (Phase Separations Ltd., Rock Ferry, Cheshire, U.K.) was used. The separations were conducted at 180°C and the gas flow rate was 35ml/min. Two isomers of $C_{20:3}$ and $C_{20:4}$ fatty acids were detected.

Fatty acids ...	Fatty acid distribution (mol/100mol)										
	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:3}$	$C_{20:3}$	$C_{20:4}$	$C_{20:4}$	$C_{20:5}$
Original phosphatidylinositol	6.7	0.5	2.0	47.1	26.6	5.1	Trace	3.0	Trace	8.2	Trace	Trace
Unfractionated dimethylphosphatidic acid	8.4	0.6	2.1	47.3	26.8	6.2	Trace	Trace	Trace	8.2	Trace	Trace
Molecular species (method 1)												
0 Penta-+hexa-enoic	—	—	—	27.0	—	—	9.0	—	42.6	—	—	21.4
1 Tetra-+penta-enoic	—	—	0.2	47.9	—	—	—	—	—	19.0	9.5	23.3
2 Tetraenoic	—	—	1.6	56.6	—	—	—	—	—	41.8	—	—
3 Trienoic	—	—	2.7	59.0	—	—	2.3	20.0	16.0	—	—	—
4 Dienoic	—	—	1.9	39.1	—	59.0	—	—	—	—	—	—
5 Monoenoic	6.1	1.6	1.6	38.8	51.8	—	—	—	—	—	—	—
6 Saturated	—	—	14.3	85.7	—	—	—	—	—	—	—	—

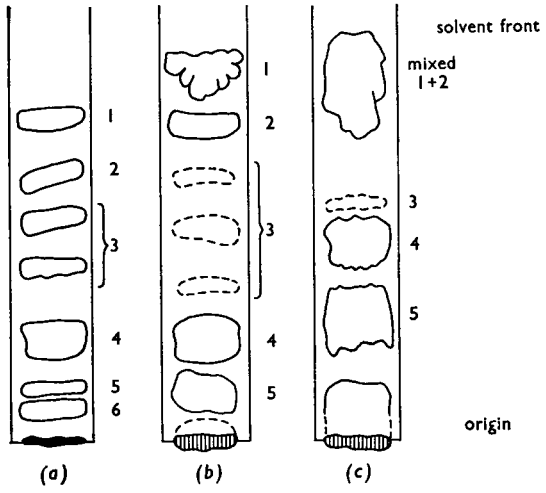

Fig. 3. *Separation of molecular species by t.l.c. on $AgNO_3$-impregnated silica gel*

Fractionation of (*a*) dimethylphosphatidic acids (method 1) from lamb liver phosphatidylinositol, and (*b*) and (*c*) methylated–triacetylated derivatives (method 2) from ox brain phosphatidylinositol by using 25 and 50% (v/v) acetone in distilled chloroform respectively. The numerals indicate the total number of double bonds in each molecular species as demonstrated by g.l.c. of their fatty acid constituents. The t.l.c. conditions employed in the separations are described in the text. Broken lines indicate minor components.

molecular species eluted from $AgNO_3$-impregnated t.l.c. plates were found unsuitable. For example, digestion of the eluted lipids with 72% (w/w) $HClO_4$ led to incomplete hydrolysis to P_i due to the methylation of the phosphate group. Similarly, chemical determination of the ester groups by the hydroxamic acid reaction was found to be subject to many interferences and was not reproducible. The above technique utilizes [^{3}H]methanol to esterify the fatty acids of the molecular species. Determination of the radioactivity by liquid-scintillation counting thus allows a direct quantitative measure of their distribution.

Table 2 shows the pattern of distribution of molecular species in lamb liver phosphatidylinositol by using the above techniques. The most predominant species is the monoenoic (60%) followed by the tetraenoic (17%) with smaller amounts of the remaining species. In some analytical determinations, the trienoic species of dimethylphosphatidic acid was fractionated into two bands as shown in Fig. 3(*a*).

Table 2. *Distribution of molecular species in lamb liver phosphatidylinositol obtained by methods* 1 *and* 2

Results are expressed as mol/100mol of the total molecular species recovered after t.l.c. on $AgNO_3$-impregnated silica gel in both methods.

	Distribution (mol/100mol)	
Molecular species	Method 1	Method 2
Monoenoic	60	58
Dienoic	6	10
Trienoic (Δ1+2)*	2	—
(Δ0+3)*	9	8
Tetraenoic	17	18
Pentaenoic	3	6
Hexaenoic	4	

* The trienoic species was sometimes fractionated into two bands that were identified by g.l.c. The degree of unsaturation of their constituent fatty acids is indicated by the numbers in parentheses.

Method 2. Fractionation of molecular species of phosphatidylinositol by masking the phosphorylinositol moiety

Acetylation of phosphatidylinositol. A solution of phosphatidylinositol (0.5–1.5mg of P in 4.2ml of chloroform) was treated with 3.3ml of acetic anhydride and 0.37ml of pyridine. These volumes could be scaled down to cope with as little as 250μg of phosphatidylinositol P. The reaction was carried out under N_2 for 2h at 37°C. In some experiments, [1-^{14}C]acetic anhydride was used to produce labelled acetylation products. The reaction was stopped by adding 2 vol. of 0.9% NaCl solution and the lower chloroform phase was further washed with 3vol. of 1M-HCl. This purification treatment removed the excess of acetylation reagents and the water soluble P-containing reaction products (17% of the original phosphatidylinositol P) to the aqueous phase.

The water soluble P-containing compound(s) was found associated with 8.5 molar equivalents of [1-^{14}C]acetic anhydride but with no long-chain fatty acids. When subjected to mild alkaline hydrolysis, the major product detected after electrophoresis was glycerylphosphorylinositol and small amounts of glycerophosphoric acid. It is therefore likely that the P-containing compound(s) in the upper phase was a highly acetylated hydrolysis product of the original phosphatidylinositol.

The rate of acetylation of phosphatidylinositol was followed by measuring the appearance of radioactivity from [1-^{14}C]acetic anhydride into the lipid products of the reaction in the lower phase (Fig. 4). The reaction was almost complete in 2h. The lipid

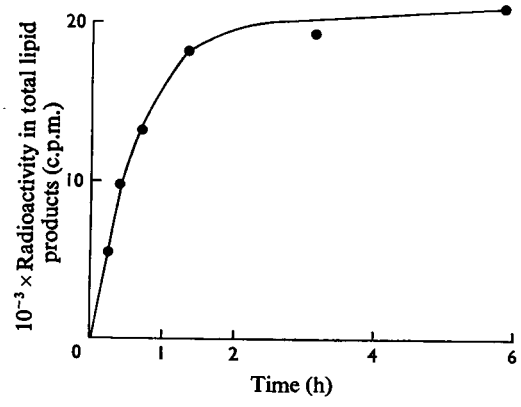

Fig. 4. *Progress of acetylation of phosphatidylinositol*

Lamb liver phosphatidylinositol (10.5μmol) was reacted with [1-^{14}C]acetic anhydride (specific radioactivity 520d.p.m./μmol) under the conditions described in text, and the appearance of radioactivity in the lipid products was followed.

products at different time-intervals were also examined by t.l.c. with two different solvent systems. In the first, chloroform–methanol–acetic acid–water (65:24:5:1, by vol.), only one product (R_F 0.82) was observed near the end of the reaction period. In the second solvent system, chloroform–methanol (7:3, v/v), about 63% of the recovered phosphorus had R_F 0.42, and 31% in a spot near the solvent front. Since only 82% of the original phosphatidylinositol P was recovered as acetylated lipid products, this meant a net conversion of 52% of the original phosphatidylinositol into the major acetylated product (R_F 0.42, Figs. 1*e* and 1*f*).

The major acetylated compound contained fatty acids similar in their composition to that of the original phosphatidylinositol (Table 4), thus indicating no significant autoxidation during the acetylation step. When the reaction was conducted in [1-^{14}C]acetic anhydride of known specific radioactivity, it was found that 3 mole equivalents were introduced into every mole of the original phosphatidylinositol (Table 3).

I.r. examination of the major acetylated product (Fig. 2*d*) showed that in comparison with phosphatidylinositol (Fig. 2*a*) the intensities of the peaks at 3300 and 1100cm^{-1} were decreased, indicating that some of the inositol hydroxyl groups were masked by the treatment. On the other hand, a peak appeared at 1360cm^{-1} corresponding to the acetyl group introduced by acetylation.

Experiments on the separation of the tri-acetylated phosphatidylinositol into molecular species by t.l.c.

Table 3. *Number of acetyl groups introduced into the major product of acetylation of phosphatidylinositol (R_F 0.42, Fig. 1f)*

[1-^{14}C]Acetic anhydride of known specific radioactivity was used, and the bound reagent/P molar ratio was determined in sample of phosphatidylinositol of different origin.

Source of phosphatidylinositol	Acetyl groups/P (molar ratio)
Lamb liver	3.24
Guinea-pig brain	3.01
Ox brain (whole)	3.13
Ox brain (white matter)	3.03
Ox brain (grey matter)	2.95

on $AgNO_3$-impregnated silica gel H proved that the product was still much too polar to allow a useful fractionation. The most favourable conditions were obtained by activating the $AgNO_3$-impregnated layers at 180°C for 3h, and by using a rather polar developing solvent (chloroform–methanol; 7:3, v/v). Even then, only four bands were obtained and from examination of their fatty acid compositions, they appeared to be incompletely resolved. This was further demonstrated when the monoenoic species of phosphatidyltri[1-^{14}C]acetylinositol was rechromatographed on a $AgNO_3$-impregnated plate under conditions identical with those described above. The radioactivity was found to trail in the manner described in Fig. 5. This difficulty is also apparent in all methods using intact phospholipids for this type of fractionation (see the Discussion section). Further masking of the polar moiety of phosphatidylinositol was therefore achieved by methylation of the phosphate group.

Diazomethanolysis of triacetylated phosphatidylinositol. Diazomethanolysis was carried out as described above for the production of dimethylphosphatidic acid.

On examination of the reaction products by t.l.c., with ether as solvent, two major products were detected and these had greater mobilities than the original triacetylated phosphatidylinositol (Fig. 1*g*). If triacetylated phosphatidylinositol is reacted in the K^+ form, the faster-moving compound was produced to a greater extent (Fig. 1*h*), whereas if it was reacted in the acid form, the slower-moving compound was produced (Fig. 1*i*).

Both methylated compounds had identical fatty acid composition, and they behaved identically on subsequent t.l.c. on $AgNO_3$-impregnated silica gel. I.r. examination showed that both products were methylated on the phosphate group but that not all

Table 4. *Fatty acid distribution in different molecular species of ox brain phosphatidylinositol fractionated by method 2*

The g.l.c. conditions were as described in Table 1. Results are expressed as mol/100 mol of total fatty acids recovered. Bands are numbered from the least to the most mobile on the $AgNO_3$-impregnated plate. The origin spot is not included. Two dienoic and two trienoic bands were combined as shown in the table. The solvent system employed in the t.l.c. step was 25% (v/v) acetone in distilled chloroform. Two $C_{20:3}$ and two $C_{20:4}$ fatty acid isomers were detected.

	Fatty acid distribution (mol/100 mol)											
Fatty acids ...	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:1}$	$C_{20:2}$	$C_{20:3}$	$C_{20:3}$	$C_{20:4}$	$C_{20:4}$	$C_{22:2}$
Whole ox brain phosphatidylinositol	6.4	Trace	Trace	40.3	10.9	Trace	Trace	Trace	0.6	Trace	42.4	Trace	Trace
Acetylated derivative	8.0	1.1	Trace	38.0	11.7	Trace	Trace	Trace	0.9	Trace	40.0	Trace	Trace
Methylated–acetylated derivative	10.1	Trace	1.3	38.6	13.3	Trace	Trace	Trace	0.6	Trace	36.0	Trace	Trace
Molecular species (method 2)													
1 Pentaenoic	7.0	1.4	0.3	21.4	22.5	—	—	—	—	—	35.7	11.7	—
2 Tetraenoic	5.7	—	0.85	38.5	4.6	—	—	—	—	—	34.5	16.2	—
3 Trienoic ($\Delta 0+3$, $\Delta 1+2$)*	14.5	1.9	1.0	27.6	14.5	—	—	9.5	17.0	14.0	—	—	—
4 Dienoic ($\Delta 0+2$, $\Delta 1+1$)*	16.0	7.0	—	12.7	44.8	8.4	0.6	3.5	—	—	—	—	7.0
5 Monoenoic	28.2	8.7	—	17.7	28.6	1.8	7.6	—	—	—	—	—	7.5

* The di- and tri-enoic molecular species were each fractionated into two bands that were combined because of their low concentrations. The presented g.l.c. analysis indicates that the original bands contained bound fatty acids whose degree of unsaturation is indicated by the numbers in parentheses.

Fig. 5. *Chromatographic behaviour of triacetylphosphatidylinositol on thin-layer plates impregnated with $AgNO_3$*

A sample of triacetylphosphatidylinositol labelled with [1-^{14}C]acetic anhydride was subjected to t.l.c. on $AgNO_3$-impregnated silica gel under the conditions described in the text. (*a*) Shows the pattern of resolution of molecular species, with numerals indicating the total number of double bonds in each band. The highly mobile monoenoic species was eluted and rechromatographed under identical conditions. (*b*) Shows the trailing of the radioactivity over the whole length of the plate.

vibrational modes of the C–O–P bond were allowed in each (see bands at frequencies 1040 and 1170 cm^{-1}, Figs. 2*e* and 2*f*). Bands corresponding to the acetyl groups were also apparent in both compounds, but again with different intensities indicating that not all modes of vibration are allowed in each. The compounds, therefore, may represent different conformational forms of the same material, selected for by the salt form of the original triacetylated phosphatidylinositol.

To produce a compound easily purified on preparative t.l.c., it is therefore preferable to methylate the acid form of triacetylated phosphatidylinositol.

T.l.c. on $AgNO_3$-impregnated silica gel G. The chromatographic behaviour of the methylated–triacetylated derivative indicates their extreme non-

polar nature. So much so, that on plates impregnated with $AgNO_3$ (6g/20g of adsorbent), only acetone–chloroform solvent mixtures afforded a reasonable separation. Two mixtures [25 and 50% (v/v) acetone in distilled chloroform] were found suitable and were used in a complementary fashion. The less polar one afforded a good separation of mono-, di- and tri-enoic species (Fig. 3*b*), whereas the more polar solvent effected a separation of the tetra- and penta-enoic species (Fig. 3*c*).

An example of the fatty acid composition of the different bands obtained after t.l.c. on $AgNO_3$-impregnated silica gel is given in Table 4 for whole ox brain phosphatidylinositol. Molecular species were clearly separated depending on their degree of unsaturation, and no trailing of the monoenoic species was exhibited (see above for the triacetylated derivative). Since in the brain the tetraenoic species is a major one it has tended to mask the pentaenoic species somewhat.

Distribution of molecular species of phosphatidylinositol from lamb liver. A sensitive method of determination of different molecular species has afforded itself in the case of the methylated–triacetylated derivative of phosphatidylinositol through the use of [1-^{14}C]acetic anhydride of known specific radioactivity in the acetylation reaction. Since the final product has been shown to possess the same fatty acid composition as the original phosphatidylinositol, and since the acetylation reaction under the conditions employed followed a fixed stoicheiometry, direct determination of the radioactivity of the different molecular species was therefore a valid method of their determination. The bands were scraped off the plates and the radioactivity was determined directly in the same liquid scintillant as described above for [^{3}H]methanol. Suitable quench curves were prepared for the interference by silica gel–$AgNO_3$.

An example of such a determination which combines results from identical runs in the two solvent systems mentioned above is given in Table 2. The pattern of distribution of molecular species of phosphatidylinositol from lamb liver by using this method agrees fairly well with that obtained from the fractionation of dimethylphosphatidic acid derived from the same sample of phosphatidylinositol (method 1).

Discussion

The analysis of molecular species of phosphatidylinositol has been achieved recently by Holub *et al.* (1970), and by Akino & Shimojo (1970) using the diglyceride acetate derivatives (Renkonen, 1967). Our methods, however, have one major advantage over theirs. The derivatives employed in the present work preserve either part or the whole of the head group. The shorter method (production of dimethylphosphatidic acid) allows the study of the independent turnover of the phosphate and fatty acid parts of the phosphatidylinositol molecule, whereas the longer method (production of methyltriacetylphosphatidylinositol) allows a similar study for all parts of the molecule.

During the preparation of this manuscript, Holub & Kuksis (1971) reported a separation of molecular species of phosphatidylinositol from rat liver by direct chromatography on thin layers impregnated with $AgNO_3$. By using highly activated plates (120°C for 20h) and a solvent system of chloroform–methanol–water, they pointed out that the conditions used were critical, and that the method failed to resolve the mono- and di-enoic species. Moreover, the monoenoic species appeared to trail along the plate thus limiting the usefulness of the method for phosphatidylinositol containing much of this species. We have demonstrated that the same situation holds even if phosphatidylinositol was only acetylated (see the Experimental and Results section). In our complete methods, not only was the monoenoic species clearly resolved, but the di- and tri-enoic species were resolved into different subspecies depending on the distribution of unsaturation between the two acyl groups of phosphatidylinositol (Fig. 3; also M. G. Luthra & A. Sheltawy, unpublished work).

The mild acetylation conditions employed in the longer method (at 37°C for 2h) produced a derivative of phosphatidylinositol in which only three hydroxyl groups of the inositol were changed. More drastic conditions (145°C for 4h; Renkonen, 1967) produce diglyceride acetates and some hydrolysis of the acyl ester linkages. This is supported by the fact that in spite of the mild conditions employed in this work, 17% of the original phosphatidylinositol P was lost as a highly acetylated water-soluble compound. However, the fatty acid composition of the remaining portion (triacetylphosphatidylinositol) was identical with that of the starting phospholipid indicating no specific loss of certain fatty acids.

The major molecular species of lamb liver phosphatidylinositol found in the present work is the monoenoic (60%) followed by the tetraenoic (17%, Table 2). Akino & Shimojo (1970) and Holub & Kuksis (1971) found that the tetraenoic species predominates (87%) in rat liver phosphatidylinositol. This is not altogether unexpected since the fatty acid composition of the unfractionated phosphatidylinositol from the two sources differ greatly. Moreover, phosphatidylcholines from the livers of different animals were also found to vary considerably in the distribution of their molecular species (Montfoort *et al.*, 1971).

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