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Alkaline *O*→*N*-transacylation

A new method for the quantitative deacylation of phospholipids

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1. Quantitative *O*-deacylation of phospholipids has been achieved by incubation with a reagent containing monomethylamine, methanol and water. The reaction is primarily an *O*→*N*-transacylation with *N*-methyl fatty acid amides being formed. 2. The reagent can be removed easily by volatilization and under defined conditions no secondary decomposition of the phosphorus-containing deacylation products occurs. 3. The water-soluble phosphorus compounds derived by deacylation of mammalian tissue *O*-diacylated phospholipids have been completely separated by a single-dimensional paper ionophoresis with a volatile pH 9 buffer. 4. The *O*-deacylated alkyl and alkenyl phospholipids have been examined by t.l.c. before and after catalytic hydrolysis with Hg^{2+}. 5. A complete analysis of rat brain phospholipids by the above methods agrees closely with that obtained by other procedures.

Alkali metal hydroxide alcoholysis was introduced some years ago as a method of deacylating phospholipids, during structural studies and to assist in the analysis of complex phospholipid mixtures (Dawson, 1954). Since that time it has been extensively used in many investigations (Dawson, 1979). In the present study quantitative deacylation has been accomplished by using monomethylamine as both alkali and acyl acceptor. This has two main advantages. First, under defined conditions secondary reactions other than deacylation are virtually eliminated. Secondly, subsequent to the reaction the excess reagent can be quickly removed by volatilization rather than by the use of ion-exchange columns. This allows for an easier examination of the plasmalogen and glycerol ether forms of phospholipids.

In addition a single-dimensional paper-ionophoretic method is described for the separation of water-soluble phosphorus-containing deacylation products that enables the true 'diacyl' glycerophospholipid content of tissue phospholipid mixtures to be directly and rapidly assessed. This has been used in conjunction with an Hg^{2+}-catalysed hydrolysis of the residual plasmalogens to examine the phospholipids present in certain rat tissues.

Methods

Preparation of phospholipid extracts for alkaline O→N-transacylation

Extraction of the lipids from tissues was carried out by any of the conventional techniques, such as by homogenization with chloroform/methanol (2:1, v/v) by the procedure of Folch *et al.* (1957). For an uncomplicated deacylation reaction it was necessary to reduce the alkaline-earth metal content (calcium, magnesium) of the extract to negligible proportions, so that the lipid preparation was more soluble in polar solvents. This was achieved by washing the extract from the procedure of Folch *et al.* (1957) with 0.2 vol. of 0.145 M-NaCl and repeating the washing of the lower phase several times with an upper phase prepared from e.g. chloroform/methanol/0.145 M-NaCl (16:8:5, by vol.). If the lipid extract was rich in acidic phospholipids, which have a specific affinity for alkaline-earth metals, it was sometimes necessary to employ a different procedure, e.g. washing with 0.1 M-HCl if plasmalogens were absent or otherwise 0.1 M-Na-EDTA or better, Na-CDTA (cyclohexane-1,2-diaminetetra-acetic acid) at neutral pH.

Preparation of monomethylamine reagent

Monomethylamine gas was passed into 40 ml of a mixture consisting of methanol/water/n-butanol (4:3:1, by vol.) contained in a graduated container in an ice bath until the volume had increased to 65 ml. Although the gas can be generated by gently warming a mixture of monomethylamine hydrochloride and NaOH it was found much more convenient to use one of the small laboratory

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cylinders of liquified gas (B.D.H., Poole, Dorset, U.K.; 180g). An anti-suck-back device or trap was essential. The reagent stored well in a stoppered bottle at −15°C. An alternative reagent was sometimes prepared from the commercially available 33% monomethylamine in ethanol solution (B.D.H.) by adding 30% by volume of water, although the rate of *O*→*N*-transacylation achieved was somewhat slower than with the above mixture.

Deacylation of phospholipids

Typically a portion of the phospholipid solution (250μg of lipid phosphorus) essentially free of bivalent metal ions (Ca^{2+} and Mg^{2+}) was evaporated to dryness in a stoppered tube and the lipid residue was then treated with 3ml of the monomethylamine reagent prepared as described above. The lipid was solubilized by warming gently and swirling. The butanol present in the reagent assisted the solubilization. Chloroform or carbon tetrachloride could be used to help to dissolve the lipid but these solvents decreased the rate of transacylation and to a limited extent interacted with the monomethylamine during the reaction. The tube's stopper was clipped on or held with a rubber band and the mixture maintained at 53°C in a water bath for 30–60min (the longer time if complete *O*-deacylation of plasmalogens and glycerol ether phospholipids was required).

The mixture was cooled in ice and 1.5ml of ice-cold n-propanol was added. The mixture was evaporated *in vacuo* using a splash head, allowing it to warm up slowly with swirling (propanol prevents bumping and frothing). When the methylamine gas had been largely removed, the mixture was taken to dryness in a water bath at 50°C. To the dry residue was added 1ml of water and 1.2ml of a mixture containing n-butanol/light petroleum (b.p. 40–60°C)/ethyl formate (20:4:1, by vol.) (the latter to neutralize any traces of monomethylamine remaining). After shaking, the upper phase was removed from the lower aqueous layer containing all the water-soluble phosphorus produced by the deacylation. The latter phase was rewashed with 0.75ml of the same solvent mixture. Butanol in the biphasic separation mixture facilitated the removal of lysoplasmalogens (alkenyl) and lysoglycerol ether phospholipids (alkyl) from the aqueous layer (Bjerve *et al.*, 1974).

Subsequent examination of deacylated phospholipids

Water-soluble phosphorus-containing products. A suitable portion of the aqueous phase was spotted on to Whatman no. 1 chromatographic paper (previously washed well with 2M-acetic acid followed by deionized water) as a narrow band. The paper was wetted with buffer and subjected to ionophoresis for 90min (55V/cm) under water-cooled white spirit (high-flash-point-petroleum fraction) as described previously (Dawson *et al.*, 1962; Dawson, 1976). The aqueous volatile buffer (pH9) used was a mixture of ammonium carbamate (mol.wt. 78.07)/ammonium acetate at 0.1–0.2M final concentrations. This buffer could be diluted with 2 vol. of water to decrease the current and heating problems in the tank, but the resultant spots tended to be less compact.

After the papers had been dried at 70°C to remove buffer components, they were sprayed with 0.25% ninhydrin in acetone and heated at 100°C for 3min to reveal amino compounds (e.g. glycerophosphoethanolamine, glycerophosphoserine). They were then sprayed with a freshly mixed reagent of 4% ammonium molybdate in 9% $HClO_4$ (1vol.) plus 7.2% $HClO_4$/conc. HCl (110:1, v/v; 3 vol.). After drying the papers at room temperature the phosphorus-containing compounds were developed as blue spots by exposure to u.v. light. For quantitative assessment they were cut out, digested and their phosphorus contents were determined (Dawson, 1976).

Lipid products. The alkenyl (plasmalogens) and alkyl (glycerol ether) phospholipids and sphingomyelin present in the butanol phase were examined by a procedure similar to that described by Dawson (1976), but simpler since *O*-deacylation was complete. Portions of the combined butanol phases containing the lipid deacylation products were examined by t.l.c. before and after catalytic mercuric hydrolysis of the plasmalogens (Norton, 1959). To one portion was added 4% by volume of a reagent containing $HgCl_2$ (0.27g) dissolved in 1ml of water plus 9ml of methanol. Hydrolysis was carried out for 10min at 37°C and the butanol layer was then washed with an equal volume of water saturated with the n-butanol/light petroleum/ethyl formate (20:4:1, by vol.). The aqueous layer containing any glycerophospho-derivatives split off from the lysoplasmalogens was sometimes examined by high-voltage ionophoresis (as above). Both the original butanol extract containing the lysoplasmalogens (alkenyl), glycerol ether phospholipids (alkyl) and sphingomyelin, and a portion of the washed butanol phase from the $HgCl_2$ hydrolysis and not containing the former were taken to dryness *in vacuo* after the addition of ethanol (4vol.) to facilitate the distillation of the butanol at low pressure. The residues were redissolved in a little chloroform and a portion was applied to a t.l.c. plate (Merck F254 silica gel). Non-polar lipids were cleared to the top of the plate by using a run with diethyl ether as developing solvent and the phospholipid chromatogram was then developed in chloroform/methanol/diethylamine/water (110:50:8:5, by vol.) or chloroform/methanol/diethylamine/conc. NH_3 solution

(110:50:10:13, by vol.). The plates were well-dried and then sprayed with phosphorus-detecting reagent (Vaskovsky & Kostetsky, 1968). It was consistently noted that any alkenyl-containing plasmalogen phospholipids gave a low phosphorus colour response with the latter reagent. The spots on the plates could be charred by heating in an oven at 110°C for 15–30min; resolution improved on cooling. For quantitative assessment the spots were scraped off and the phosphorus was determined (Dawson, 1976).

Results

Transacylation induced by organic bases

Early attempts were made to obtain quantitative transacylation from phospholipids by using NH_3 as acceptor. Ovophosphatidylcholine was completely deacylated when heated in a sealed tube for 18h at 43°C or 2h at 50°C with a mixture of conc. NH_3 solution (0.880 sp.gr.) and methanol (1:1, v/v). No cyclic glycerophosphate was formed. However, other phospholipids, e.g. phosphatidylinositol, were more resistant to deacylation, probably because they were insoluble in the reaction mixture. A mixture of conc. aq. NH_3 and conc. methanolic NH_3 (1:4, v/v) completely deacylated both phosphatidylcholine and phosphatidylinositol when heated with these phospholipids in a sealed tube for 1h at 100°C.

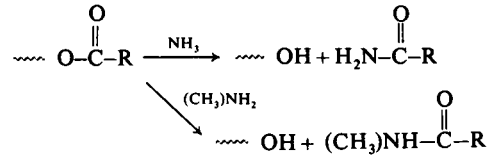
Monomethylamine proved to be much superior to NH_3 as a reagent for inducing *O*→*N*-transacylation, and in addition although it was volatile enough to be easily removed after completion of the reaction, the monomethylamine-containing reagent could nevertheless be incubated with the lipids at 53°C in a well-stoppered tube without serious loss of base. Other bases tried were not so effective and although precise quantitative values were not obtained, the ability to deacylate phospholipids was in the order monomethylamine > monoethylamine > diethylamine > dimethylamine > isopropylamine > aniline. The deacylating ability of isopropylamine was very poor and of aniline non-existent.

The deacylating ability of monomethylamine/methanol (5:4, v/v) was enhanced by adding water. Thus with a rat brain lipid extract, the addition of 1 vol. of water (i.e. monomethylamine/methanol/water, 5:4:1) released 45% of the lipid phosphorus as water soluble after 8min incubation at 53°C, 2 vol. (i.e. 5:4:2) produced 58% and 3 vol. (i.e. 5:4:3) 62%. The maximum liberation obtained with longer times of incubation was 69%, the residual 31% representing phospholipids containing hydrophobic groups other than *O*-acyl residues.

From such observations the optimum reagent for providing good deacylation rates and reasonable solubility of the phospholipid extracted was chosen as a mix of monomethylamine/methanol/water/butanol (5:4:3:1, by vol.), butanol being present to assist in the 'solubilizing' of the phospholipids. At 53°C this produced complete deacylation of the *O*-diacyl groups in a mixture of rat brain phospholipids within 20min. The plasmalogens and glycerol ether phospholipids, which are more resistant to deacylation (Hanahan & Watts, 1961; Ansell & Spanner, 1963; Renkonen, 1963), took up to 1h before being completely deacylated. Methyl and butyroyl fatty acid esters were decomposed more slowly than the phospholipids and cholesterol stearate was resistant to deacylation.

Nature of the reaction

Both with NH_3 and monomethylamine the predominant reaction was an *O*→*N* transacylation forming the fatty acid amide or its monomethyl derivative.



The soluble lipid products were separated by t.l.c. [silica gel; diethyl ether/methanol (10:3, v/v) as solvent system] and when located with I_2 vapour gave the following R_F values: *N*-acylammonia, 0.25; *N*-acylmethylamine, 0.31; non-esterified fatty acid 0.70; fatty acid methyl ester, 0.79. The identities of the main *N*-containing products were checked by gas chromatography (column, 3% SE30 on diatomite C'Q'; 186°C) with methyl palmitate as the donor acyl group. With NH_3, palmitamide $R_{\text{methyl palmitate}} = 2.41$ and with monomethylamine, *N*-methylpalmitamide $R_{\text{methyl palmitate}} = 2.77$ were produced, which ran identically with synthetic standards of these substances. Using the monomethylamine reagent, minimal transacylation from the phospholipid to water, producing fatty acid, and to methanol, forming fatty acid methyl esters, occurred. Presumably such transfers were catalysed by the presence of the organic base. However, with the excess base present both of these were converted into the *N*-acyl derivative as the reaction proceeded.

Under the reaction conditions specified there was little evidence of any secondary decomposition of the phosphorus-containing products. Glycerol cyclic phosphate, a minor product of alkali-metal hydroxide/alcohol deacylation of phospholipids (Marou & Benson, 1959), was not detected in significant amounts. Phosphatidylinositol, which yielded a diversity of water-soluble phosphate esters on NaOH-catalysed methanolysis (Dawson *et al.*, 1962), gave glycerophosphoinositol exclusively, providing it was in the form of its ammonium. sodium or potassium salt. As the barium or calcium salt it

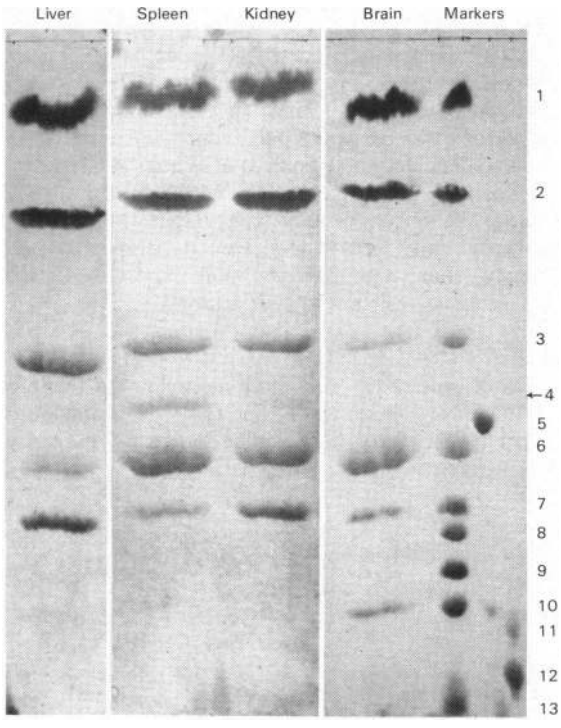


Fig. 1. *Paper ionophoresis of water-soluble phosphorus compounds released by deacylation of rat tissue phospholipids*

The water-soluble deacylation products were prepared as stated in the Methods section. They were spotted on washed Whatman no. 1 paper and ionophoresis was carried out (90 min at 55 V/cm) after the paper had been wetted with ammonium carbamate/ammonium acetate buffer (pH 9). The dried paper was sprayed to detect phosphorus. The Figure is a composite prepared from ionophoretograms run at different times. Marker lanes contained a mixture of various phosphate esters. Identity of compounds with anodic mobility relative to P_i: (1), glycerophosphocholine ($M = 0.09$); (2), glycerophosphoethanolamine ($M = 0.25$); (3), glycerophosphoinositol ($M = 0.46$); (4), glycerophosphoglycerol ($M = 0.55$); (5), inositol 1:2-cyclic phosphate ($M = 0.57$); (6), glycerophosphoserine ($M = 0.62$); (7) bis(glycerophospho)glycerol ($M = 0.71$); (8), inositol 2-phosphate ($M = 0.76$); (9); glycerol 1:2-cyclic phosphate ($M = 0.80$); (10), glycerol 1-phosphate ($M = 0.87$); (11), inositol bisphosphate ($M = 0.86$); (12), inositol trisphosphate ($M = 0.93$); (13), P_i ($M = 1.0$). M is the mobility relative to the position of P_i. Other phosphate esters, which are not shown in the Figure, have the following mobilities: phosphocholine, 0.50; phosphoethanolamine, 0.57; glycerophosphoethanol, 0.62; glycerophosphomethanol, 0.66; galactose 6-phosphate, 0.71; inositol 1-phosphate, 0.75; glycerol 2-phosphate, 0.90; glycerophosphoinositol phosphate, 0.84; glycerophosphoinositol bisphosphate, 0.87; glycerol bisphosphate, 0.98.

gave a number of products including glycerophosphoinositol, phosphoinositol, glycerophosphate and glycerol cyclic phosphate.

Examination of rat tissue phospholipids

As a test of the new method of phospholipid deacylation we examined the phospholipids extracted from rat brain, liver, kidney and spleen. After the reaction, the water-soluble phosphorus products were resolved by a single-dimensional paper ionophoretic technique employing volatile buffer at pH 9. The individual components were well separated and there was no evidence of any secondary decomposition of the initial deacylation products (Fig. 1).

In addition, some of the hydrophobic phospholipid residues remaining after deacylation were

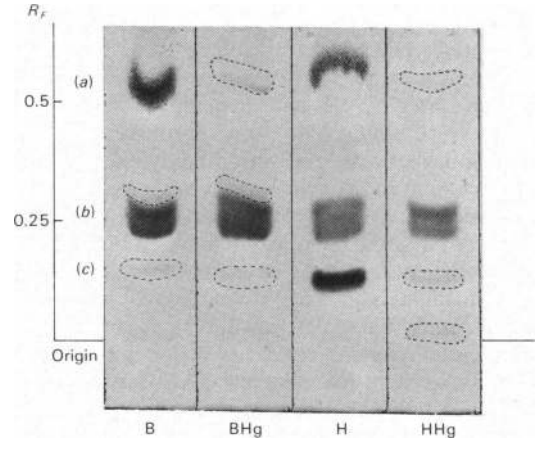


Fig. 2. *T.l.c. of phospholipids remaining solvent-soluble after N-transacylation*

Lipids extracted from sheep brain (B) and heart (H) were deacylated with methylamine for 1 h as described in the text. The phospholipids remaining soluble in the butanol layer were separated by t.l.c. either directly (B and H) or after $HgCl_2$ hydrolysis (BHg and HHg). (*a*) Alkenyl and alkylglycerophosphoethanolamine; (*b*), sphingomyelin; (*c*), alkenyl- and alkyl-glycerophosphocholine. The brain is rich in ethanolamine plasmalogen, whereas choline plasmalogen is virtually absent. The heart contains both ethanolamine and choline plasmalogen. The lyso-compounds (alkenyl) formed from them by *N*-transacylation are decomposed by the $HgCl_2$ hydrolysis leaving trace residues of the alkyl-glycerophosphoethanolamine or alkyl-glycerophosphocholine derived from the glycerol ether phospholipids. The sphingomyelin spot is partially split into two major components and in brain a third minor fast-running component was also seen. G.l.c. analysis indicated that in brain sphingomyelin the predominant fatty acid in the slower running major spot is stearic acid and the faster running major spot nervonic acid, in line with the observations of Eldin & Sloane-Stanley (1964). Broken line outlines very faint spots on the original chromatogram.

Table 1. *Analysis of phospholipids extracted into neutral chloroform/methanol solvent from whole rat brain*
All values are expressed as percentage of total lipid phosphorus.

		Present results		Results of Wuthier (1966)	Results of Wells & Dittmer (1967)	
Glycerophospholipid (choline)	Phosphatidylcholine	33.9			36.8	
	Choline plasmalogen	1.0	36.7	36.8	0.5	37.3
	Alkyl (glycerol ether)	1.8				
Glycerophospholipid (ethanolamine)	Phosphatidylethanolamine	15.4			16.0	
	Ethanolamine plasmalogen	21.3	38.2	36.4	19.4	37.0
	Alkyl (glycerol ether)	1.5			1.6	
Phosphatidylserine		13.0		11.8	12.8	
Phosphatidylinositol		3.2		3.1	3.3	
Bis(phosphatidyl)glycerol		1.7		2.2	1.8	
Phosphatidic acid		2.1		2.3	2.0	
Sphingomyelin		5.1		5.7	5.7	

separated by t.l.c., both before and after catalytic $HgCl_2$ hydrolysis (Fig. 2). It is clear that under the conditions specified *O*-deacylation of the plasmalogen and glycerol ether phospholipids was complete. $HgCl_2$-catalysed hydrolysis removed the alkenyl groups from the plasmalogens, leaving the alkyl-containing phospholipid residues and sphingomyelin. Examination by ionophoresis of the water-soluble phosphorus products formed from the plasmalogens showed that these were exclusively the expected glycerophospho-derivatives of ethanolamine and/or choline. With the rat brain phospholipids a complete distribution of phosphorus contained in the phospholipids was obtained. This agrees very well with previous analyses obtained by workers using quite different techniques (Table 1). Thus Wuthier (1966) separated the phospholipids by two-dimensional chromatography on silica gel-impregnated paper, which cannot resolve the diacyl, plasmalogen and glycerol ether forms. Wells & Dittmer (1967) decomposed the phospholipids by alkaline and acid treatment and used ion-exchange and silicic acid columns to separate the water-soluble and lipid products respectively.

Discussion

Monomethylamine would appear to be an excellent reagent for the deacylation of phospholipids. It has two advantages over alkali-metal hydroxides, which can be used to catalyse transacylation to methanol or ethanol (Dawson, 1954). First, the reaction is not complicated by secondary decomposition of the deacylated phosphorus products. Secondly, the base, since it is volatile, is easy to eliminate from the reaction mixture, and consequently it is unnecessary to remove the alkali with ion-exchange resins to prevent interference with subsequent chromatography and ionophoresis of the products. The method seems to be applicable to all the phospholipids present in mammalian tissues, and more recently we have applied it to certain complex bacterial phospholipids with equal success.

Although organic bases have been tried before as reagents for catalysing the transfer of acyl groups from phospholipids to alcohols, results with diethylamine (Dawson, 1960) and triethylamine or triethanolamine (Brockerhoff, 1963) have been disappointing, with only low yields of water-soluble phosphorus. On the other hand, quaternary ammonium compounds with their higher dissociation constants are reasonably effective (Brockerhoff, 1963; Brockerhoff & Yurkowski, 1965), but have the disadvantage of being involatile. The success of the present method of achieving complete deacylation is due to the general effectiveness of monomethylamine compared with the nitrogen-containing bases previously employed and its use in very high concentration and for a longer time at a higher temperature. The base is not only functioning as a catalyst by raising the pH, but also by acting as a receptor for the acyl groups transferred. That the dissociation constant is not the only factor involved is shown by isopropylamine, which although having the same basicity as methylamine is very ineffective as a deacylation reagent.

The complete separation of the water-soluble deacylation products by a single-dimensional paper-ionophoretic procedure provides a rapid method for resolving these compounds that is much simpler and less time consuming than the previous two-dimensional paper separations (Dawson, 1960; Dawson *et al.*, 1962). It is based on a technique described by Abdel-Latif & Abood (1965), who used a bicarbonate buffer at pH 10.9, but the present method gives more discrete resolutions and the buffer, being volatile, can largely be removed from the paper.

The techniques described give a rapid method of investigating the phospholipid composition of biological materials, which supplements and comple-

ments those achieved by other procedures. Use of a chemical method for distinguishing between the diacyl, alkenyl-acyl and alkyl-acyl forms of each phospholipid seems to be essential since direct separations by t.l.c. or column chromatography are at the best rudimentary and often non-existent.

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