# Chromatographic separation of plasmalogenic, alkyl-acyl, and diacyl forms of ethanolamine glycerophosphatides

OSSI RENKONEN

Department of Serology and Bacteriology, University of Helsinki, Helsinki, Finland

ABSTRACT The plasmalogenic, alkyl-acyl, and diacyl forms of ethanolamine glycerophosphatides were completely separated from each other as methylated dinitrophenyl derivatives by thin-layer chromatography on Silica Gel G. The relatively high resolving power needed was obtained by multiple unidimensional development with solvents that give very low mobility to the lipids. Under these conditions the plasmalogens moved fastest, the alkyl-acyl lipids were intermediate, and the diacyl lipids were the slowest. The presence of all these forms of lipids in the ethanolamine phosphatides of hen's eggs, ox brain, and human blood plasma could be directly demonstrated with the new method.

KEY WORDS ethanolamine glycerophosphatides · *N*-dinitrophenyl-*O*-methyl derivatives · thin-layer chromatography · multiple development · separation · plasmalogenic · alkyl-acyl · diacyl phospholipids

SUITABLE NONPOLAR derivatives of phospholipids and glycolipids can be separated by adsorption chromatography far more effectively than the native lipids themselves (1). For instance, many different molecular species of egg phosphatidyl ethanolamines have been separated as *O*-methylated and *N*-dinitrophenylated derivatives on silver nitrate-impregnated Silica Gel G (2). Furthermore, in this nonpolar form the egg lipids were separated by chromatography into the phosphatidyl ethanolamines proper (Fig. 1, I) and the accompanying 1-alkyl 2-acyl derivatives (Fig. 1, II) (2). The present report extends these findings and shows that the methylated DNP derivatives of ethanolamine plasmalogens, the 1-alk-1′-enyl 2-acyl derivatives, (Fig. 1, III) can also be separated from the corresponding diacyl as well as the alkyl-acyl lipids.

Abbreviations: DNP, dinitrophenyl; GPE, glycerophosphoryl ethanolamine; DEAE, diethylaminoethyl; BHT, 2,6-di-*tert*-butyl-*p*-cresol; TLC, thin-layer chromatography.

## MATERIALS AND METHODS

Glycerophosphoryl ethanolamine lipids (GPE lipids) of ox brain were isolated by combined use of DEAE-cellulose and silicic acid columns essentially as described elsewhere (3, 4); the solvents used contained 50 mg/liter of 2,6-di-*tert*-butyl-*p*-cresol (BHT) as antioxidant (5).

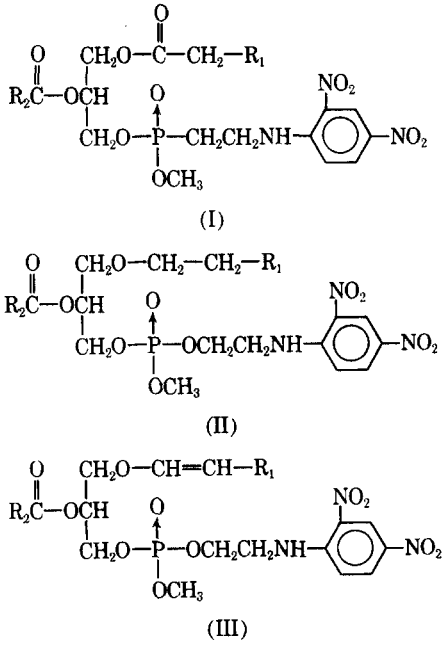


FIG. 1. Methylated DNP derivatives of subclasses of ethanolamine glycerophosphatides. I, phosphatidyl ethanolamines; II, alkyl-acyl ethanolamine glycerophosphatides; III, alkenyl-acyl ethanolamine glycerophosphatides (ethanolamine plasmalogens).

Almost pure phosphatidyl ethanolamines were obtained by silicic acid chromatography of egg lipids; the contaminants were small amounts of other GPE lipids. A synthetic sample of 1,2-dipalmitoyl GPE (Fluka AG, Buchs, Switzerland) was also available; it was purified on a column of silicic acid from which the pure lipid was eluted with chloroform–methanol 4:1.

A concentrate of native ethanolamine plasmalogens was prepared from brain GPE lipids by selective alkaline deacylation as described elsewhere (4); this treatment deacylates the phosphatidyl ethanolamines much faster than the plasmalogens.

A synthetic sample of 1-hexadecyl 2-stearoyl GPE was obtained from Dr. T. H. Bevan, Bristol, England. Before use, the sample was passed through a column of silicic acid in the same way as the 1,2-dipalmitoyl GPE.

Lysophosphatidyl ethanolamine was obtained by mild acid treatment of ox brain GPE lipids essentially as described by Debuch (6). This experiment also gave a preparation of "plasmalogen-less" phosphadityl ethanolamines; the sample could be used later with advantage to show the presence of alkyl-acyl GPE in brain lipids.

GPE lipids of human serum were isolated as described previously (7).

The GPE lipids were dinitrophenylated and methylated by a micromodification of the procedure of Collins (8). Samples of 0.5–1 mg were dissolved in 2 ml of benzene; 40 μl of triethylamine and 4 μl of fluoro-2,4-dinitrobenzene were added. After 2 hr at 20°C the reaction mixtures were evaporated to dryness and the excess reagent was removed as described by Collins. The dinitrophenylated lipids were methylated by dissolving them in chloroform and adding an excess of 0.1 M ethereal diazomethane. After 30 min at 20°C the excess reagent was evaporated off.

The methylated dinitrophenyl derivatives of egg GPE lipids were fractionated by preparative TLC as described elsewhere (2). This fractionation separated the derivatives of phosphatidyl ehanolamine from those of the 1-alkyl 2-acyl lipids.

Preparative TLC of methylated DNP derivatives of ox brain GPE lipids was carried out by using BHT-containing chloroform–methanol 99:1 on Silica Gel G, E. Merck AG, Darmstadt, Germany. Samples of about 6 mg were fractionated on layers 200 × 200 × 0.25 mm. The yellow lipid zones were visible to the naked eye, and no spray reagents were needed to detect them.

Dimethyl acetals and methylated DNP derivatives of lysophosphatidyl ethanolamines were identified by TLC essentially as described elsewhere (9); the Silica Gel G plates were developed four times with pure chloroform.

Separation of all three subtypes of GPE lipids by TLC was rather difficult, even when the methylated dinitrophenylated derivatives were used, but it could be achieved by multiple development with solvents that give very low mobility to the lipids. Mixtures of hexane and chloroform or of toluene and chloroform were suitable when used with Silica Gel G plates. BHT was added to the solvents at a concentration of 50 mg/liter to protect the samples against autoxidation during the drying periods between successive solvent ascents (10). The lipids were applied to the plates in 5 μl of hexane; the use of the nonpolar solvent for the application was essential for good resolution. The yellow color of the DNP lipids aided greatly in the development of the plates as the resolution obtained was clearly visible at all stages of the fractionation. The plates were nevertheless sprayed with Ziminski and Borowski's corrosive spray, which consists of 20% aqueous ammonium bisulfate (11).

The IR spectra were obtained by means of a Perkin-Elmer Infracord spectrophotometer, type 137.

Methylated dinitrophenylated GPE lipids were partially hydrolyzed with crude pancreatic lipase essentially in the same manner as diglyceride acetates (9).

Mild acid methanolysis of methylated dinitrophenylated GPE lipids was carried out as described earlier (4), but the procedure was adapted to a microscale as follows. The lipid sample of about 100 μg was dissolved in 75 μl of chloroform in a stoppered tube (50 × 4 mm). 75 μl of 0.1 N hydrochloric acid in methanol–water 99:1 was added and the mixture was kept at 20°C for 60 min. At the end of the reaction 75 μl of methanol–water 1:99, 25 μl of methanol, and 125 μl of chloroform were added. The system now contained chloroform, methanol, and water in the ratio 8:4:3. After equilibration of the two phases the chloroform layer was isolated and washed. The solution was finally evaporated to dryness and the lipids were dissolved in hexane for analysis by TLC.

## RESULTS AND DISCUSSION

### *Separation of Derivatives of Plasmalogens and of Diacyl GPE*

Two main fractions were observed when methylated dinitrophenylated ox brain GPE lipid was chromatographed on Silica Gel G with chloroform–methanol 99:1 (Fig. 2). The faster component appeared to consist mainly of the derivative of plasmalogens and the slower one of the diacyl lipids. This was suggested by their chromatographic similarity to model compounds, namely the methylated DNP derivative obtained from a concentrate of native ethanolamine plasmalogens and that from phosphatidyl ethanolamines.

Preparative TLC of a 45.2 mg sample of the methylated DNP derivative of ox brain GPE lipid gave 9.5

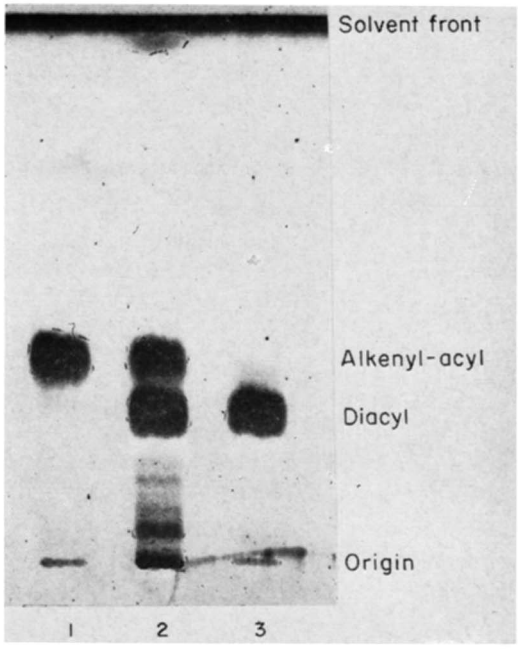


FIG. 2. Separation by TLC of ethanolamine plasmalogen and phosphatidyl ethanolamine as methylated DNP derivatives. Solvent: chloroform–methanol 99:1. Samples: *1*, the plasmalogenic derivative isolated by preparative TLC; *2*, ox brain GPE lipids prior to preparative TLC; *3*, the diacyl lipid after preparative TLC.

mg of the fast-running material and 23.5 mg of the slower component; both fractions appeared as pure lipids on repeated TLC (Fig. 2). Another, similar experiment gave 8.3 mg of the fast and 22.0 mg of the slow material. The yield of the plasmalogens was smaller than that predicted from their concentration in the original GPE lipids; it was possible to explain this discrepancy (see below) as due to the formation of some artifactual by-product from the plasmalogens which chromatographs together with the derivative of phosphatidyl ethanolamines.

The plasmalogenic nature of the fast-moving component was confirmed by its IR spectrum (Fig. 3). We observed a clear peak at 6.0 $\mu$; this peak was not present in the spectrum of the methylated dinitrophenylated 1-alkyl 2-acyl lipids (Fig. 4) or in that of the corresponding phosphatidyl ethanolamine derivatives (Fig. 5). The absorption at 6.0 $\mu$ is characteristic of the alkenyl ether group of plasmalogens (12, 13). The rather complex spectra shown in Figs. 3–5 also confirm that the ratio of carboxylic ester to phosphorus in the diacyl lipid was much higher than (theoretically twice as high as) in the plasmalogen and in the alkyl-acyl lipid. This is shown, for example, by the comparison of the peaks of P–O–C

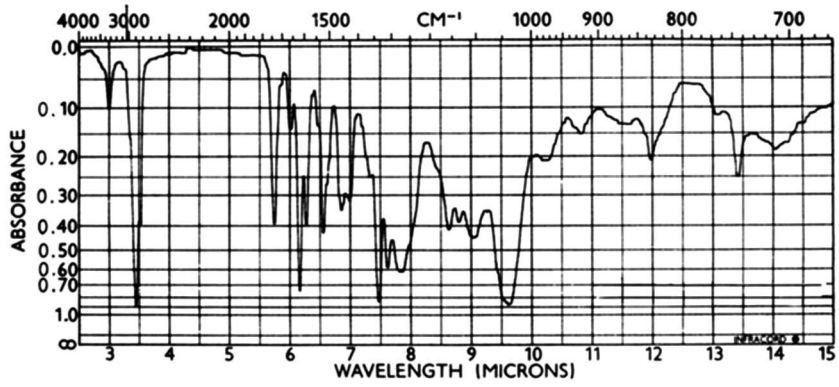


FIG. 3. IR spectrum of methylated DNP ethanolamine plasmalogens from ox brain. (Film on KBr disc.)

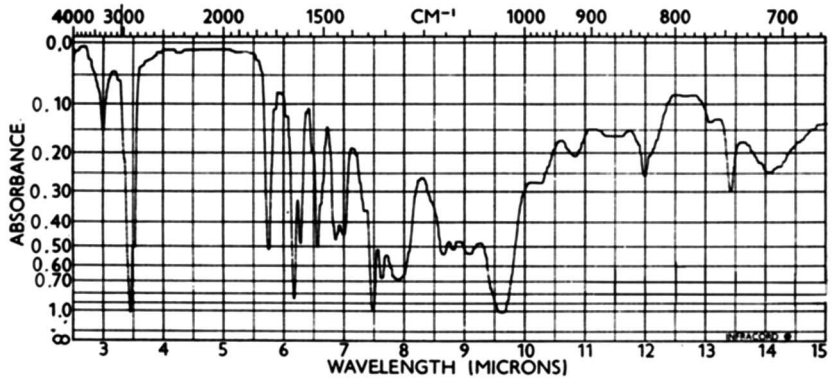


FIG. 4. IR spectrum of methylated DNP derivative of alkyl-acyl GPE from hens' eggs. (Film on KBr disc.)

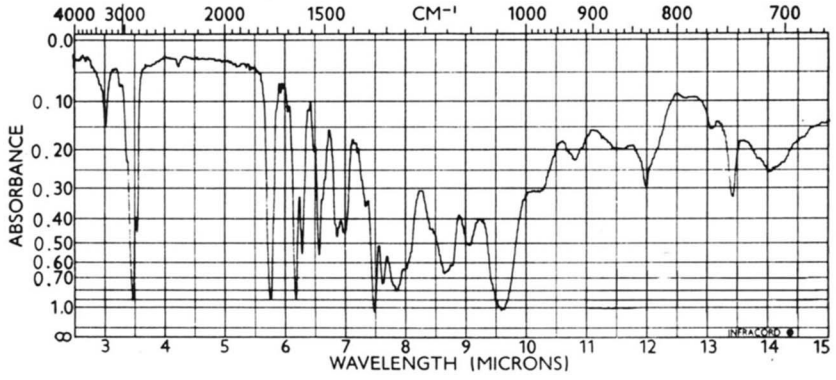


FIG. 5. IR spectrum of methylated DNP phosphatidyl ethanolamines from hens' eggs. (Film on KBr disc.)

vibration at 1040 $cm^{-1}$ with the ester bands at 1740 $cm^{-1}$ in the three spectra.

Chemical evidence was also obtained for the plasmalogenic nature of the fast-moving component shown in Fig. 2. The material released dimethyl acetals as well as methylated dinitrophenylated lysophosphatidyl ethanolamines when subjected to mild acid methanolysis; the reaction products were identified by TLC. In clear contrast to the plasmalogenic derivative the corresponding diacyl lipid and the alkyl-acyl lipid appeared to be quite stable under the acidic conditions used. Enzymic hydrolysis too provided confirmatory evidence. The fast component did not release monoacyl derivatives when treated with crude pancreatic lipase, but the slower component did. This shows that the fast component did not carry an acyl group on C-1 of glycerol. The plasmalogenic and diacyl forms of phoshatidic acid dimethyl esters also behave like this upon lipase hydrolysis (14).

All these observations suggest that ethanolamine plasmalogens can be completely separated from phosphatidyl ethanolamines when in the form of methylated DNP derivatives. This separation has not previously been achieved with any native glycerophosphatide. However, we have recently separated plasmalogens and diacyl glycerophosphatides by using other nonpolar derivatives, namely the dimethyl phosphatidates (14) or diglyceride acetates (9).

### *Separation of All Three Subclasses*

It remained to find conditions where all three subclasses of methylated and dinitrophenylated GPE lipids could be separated from one another. After considerable effort such conditions were found by subjecting the lipids to multiple unidimensional development with hexane–chloroform or toluene–chloroform mixtures on Silica Gel G plates. Fig. 6 gives an example of this; the three lipids were clearly separated when applied individually, and also when applied as a mixture. The derivatives of plasmalogens moved fastest, those of alkyl-acyl lipids were intermediate, and the diacyl lipids were the slowest. This separation equals that obtained recently with the different types of diglyceride acetates in our laboratory (9), but the methylated DNP derivatives have the advantage that the polar groups of the original phosphatides have not been eliminated.

It is remarkable that the plasmalogens run ahead of the alkyl-acyl lipids. Usually, an olefinic bond retards the movement of the lipids on silica gel (15), but in this case the reverse seems to be true. One possible explanation is that the alkenyl ether group of the plasmalogens tends to "interact" with another polar group of the same molecule under the conditions used here.

### *Natural Mixtures*

The new TLC system was also applied to some natural mixtures. Fig. 7 confirms many earlier findings (4, 16) in showing that ox brain GPE phosphatides contain all three types of lipids. Sample *1* in Fig. 7 represents methylated DNP derivatives from total GPE lipids; the bulk of the plasmalogenic lipids had first been removed from sample *3*. The spot of the alkyl-acyl lipids, which is barely distinguishable in the total GPE lipids, is quite clear in the "plasmalogen-less" preparation. Similar experiments confirmed the presence of alkyl-acyl lipids in human plasma GPE-lipids, where it was found some time ago by other methods (7).

Fig. 8 shows that the GPE lipids of hens' eggs contain traces of plasmalogens in addition to the alkyl-acyl and diacyl lipids. Sample *1* represents here the methylated DNP derivatives of alkyl-acyl lipids, but the principal component is accompanied by faint spots of the same mobility as the plasmalogens and diacyl lipids. Sample *2* shows the same lipid after mild acid treatment; the fast-running component has disappeared, which establishes further its plasmalogenic nature.

### *Formation of Artifacts with Plasmalogens*

The preparation of the methylated DNP derivatives seemed to proceed without complications in the case of

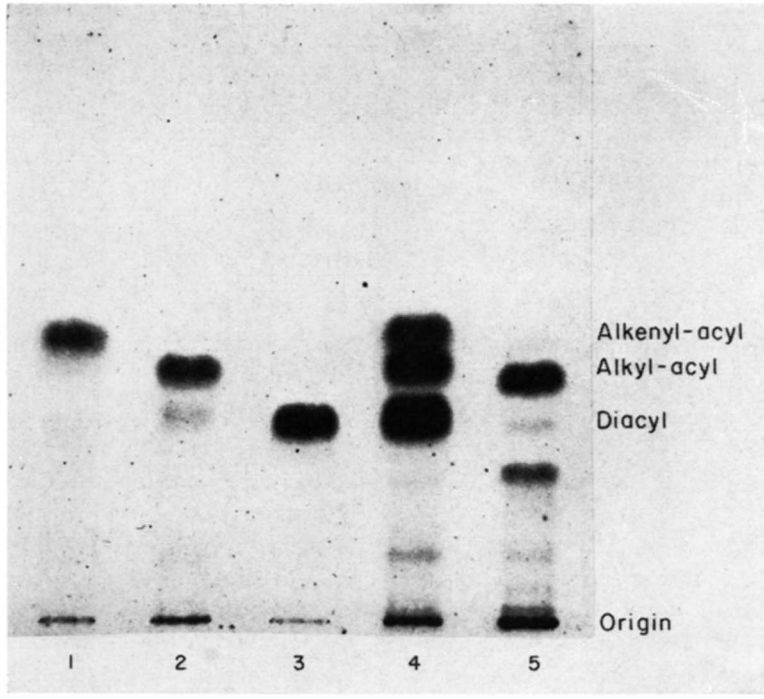


FIG. 6. TLC separation of methylated DNP derivatives of the three forms of GPE lipids. Solvent: five successive ascents of hexane–chloroform 4:6 followed by five ascents of toluene–chloroform 4:6. Samples: *1*, derivatives of plasmalogens from ox brain; *2*, derivatives of alkyl-acyl lipids from hens' eggs; *3*, derivatives of diacyl lipids from hens' eggs; *4*, mixture of *1*, *2*, and *3*; *5*, derivative of 1-hexadecyl 2-stearoyl GPE.

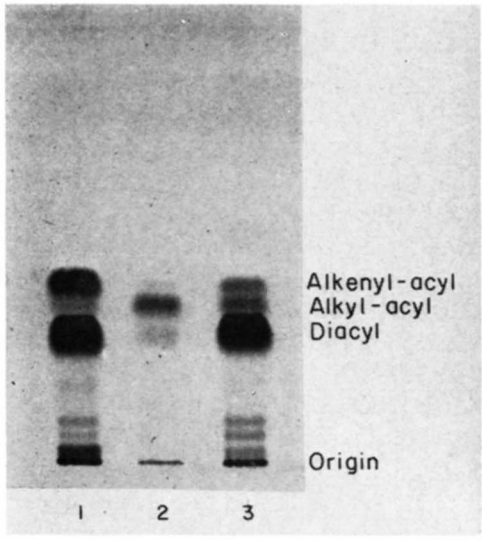


FIG. 7. Demonstration of alkyl-acyl GPE in ox brain lipids by TLC of methylated DNP derivatives. Solvent: as in Fig. 6. Samples: *1*, methylated DNP derivatives of ox brain GPE lipids; *2*, derivatives of alkyl-acyl lipids from hens' eggs; *3*, derivatives of "plasmalogen-less" (mild acid treated) GPE lipids of ox brain.

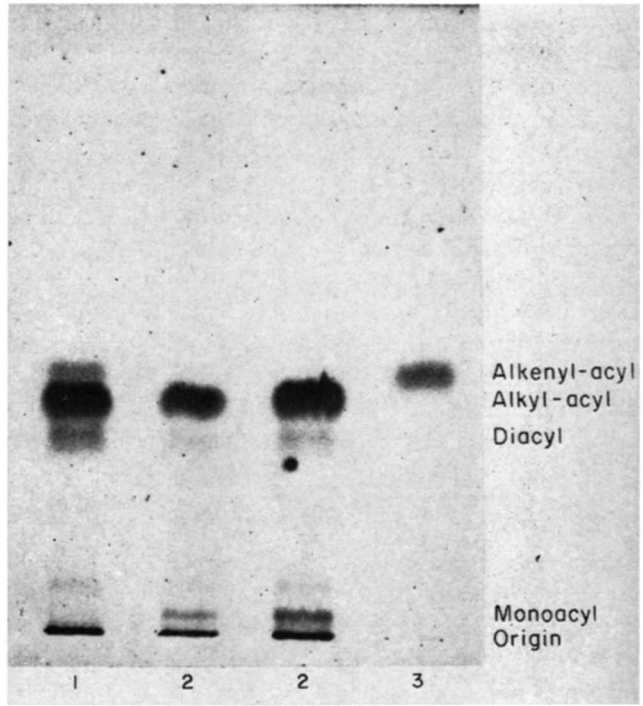


FIG. 8. Demonstration of plasmalogens in GPE lipids of hens' eggs by TLC of methylated DNP derivatives. Solvent: eight ascents with hexane–chloroform 3:7. Samples: *1*, "alkyl-acyl" lipids of hens' eggs (the sample is contaminated by plasmalogens and diacyl lipids); *2*, sample *1* after mild acid methanolysis (plasmalogen spot absent); *3*, alkenyl-acyl lipid from ox brain.

the diacyl and alkyl-acyl forms of GPE lipids. In the plasmalogens, however, an unidentified product was formed in addition to the normal derivative. On TLC the unidentified lipid ran just behind the normal diacyl derivative, from which it was very difficult to separate

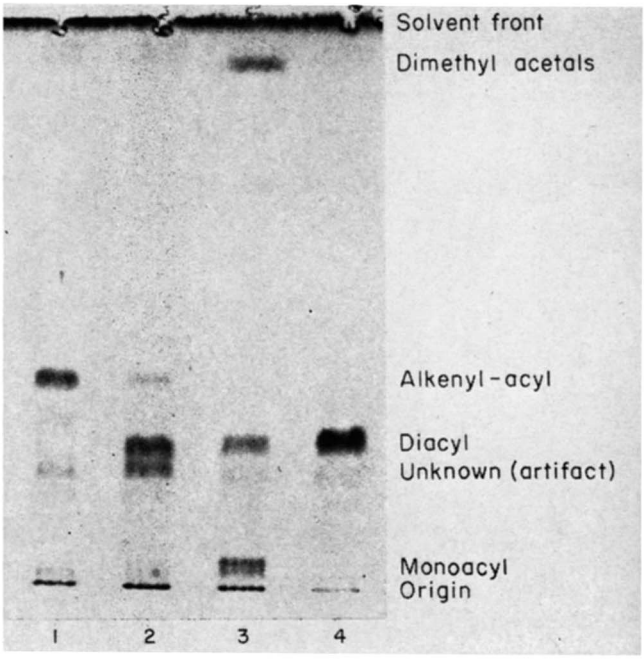


FIG. 9. Demonstration of artifact from plasmalogens by TLC. Solvent: 20 ascents with toluene–chloroform 2:8. Samples: *1*, methylated DNP derivative of "pure" ethanolamine plasmalogens; *2*, methylated DNP derivatives of GPE-lipids of ox brain; *3*, sample *2* after mild acid methanolysis; *4*, derivative of diacyl lipids from hens' eggs.

(Fig. 9). Upon mild acid treatment this material was destroyed, and two products were generated which resembled dimethyl acetals and methylated DNP derivates of lysophosphatidyl ethanolamine on TLC. The unknown material was probably generated during the methylation of the dinitrophenylated GPE lipid, as a similar by-product was resently observed in diazomethanolysates of choline plasmalogens, but not in those of other lecithins (14). This would imply a reaction between diazomethane and the reactive alkenyl ether group of the plasmalogens. The surprisingly small yield of the plasmalogenic derivative—and conversely the high yield of the diacyl fraction—obtained in the preparative TLC of the derivatives of ox brain GPE lipids was probably caused, at least partially, by this reaction.

The findings discussed in this paper provide an example of the advantages of converting polar lipids into non-polar derivatives prior to adsorption chromatography, and in addition demonstrate that very high resolution can be obtained by multiple-development TLC.

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