steroid dehydrogenase and a $\Delta^5$-3-ketosteroid isomerase. The $\Delta^5$-3$\beta$-hydroxysteroid dehydrogenase(s) involved requires a pyridine nucleotide as cofactor and the results make it probable that the enzyme(s) utilizes NAD preferentially. Thus, if the preference of pyridine nucleotide observed were due to an activation of the $\Delta^5$-3-ketosteroid isomerase(s) similar to that described by Oleinick and Koritz [9] for the $\Delta^5$-3-ketosteroid isomerase(s) in rat adrenal small particles, one would expect an accumulation of 7α-hydroxycholest-5-en-3-one in incubations with NADP. No significant amounts of labeled material with chromatographic properties of this compound could be detected. The present results do not provide any information concerning the existence of an activation of the $\Delta^5$-3-ketosteroid isomerase(s) involved by diphosphopyridine nucleotides as described by Oleinick and Koritz.[9] To detect such an activation one would have to have access to the substrates for this enzyme(s), *i.e.* 7α-hydroxycholest-5-en-3-one and 7α,12α-dihydroxycholest-5-en-3-one.

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# Analysis of Individual Molecular Species of Phospholipids

## V. Separation of Dinitrophenylated and Methylated Ethanolamine Phosphatides of Hens' Eggs*

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Different molecular species of nonpolar phospholipid derivatives are easier to separate from each other by liquid chromatography than the original phosphatide molecules. For instance diglyceride acetates,[1,11] ceramide diacetates,[2] free,[3] and tritylated diglycerides,[4] and dimethyl phosphatidates [5,6] derived from native phospholipids have been separated into many subfractions. So far all these derivatives have been obtained by removing the polar groups of the phosphatides, which has limited the value of the approach. The present report shows that ethanolamine glycerophosphatides converted into nonpolar form by dinitrophenylation and methylation, *i.e.* by "masking" the polar groups instead of removing them, are also well resolved by silica gel chromatography.

Glyceryl-phosphoryl-ethanolamine lipids (GPE-lipids) were isolated from eggs by chromatography on silicic acid. The sample was treated with 1-fluoro-2,4-dinitrobenzene and the reaction product was methylated with diazomethane as described by Collins.[7] Thin layer chromatography (TLC) of the resulting lipid revealed a yellow double-spot. The two components were isolated (Fig. 1), and the slower moving fraction (92 %) was identified as N-dinitrophenyl-O-methyl-phosphatidyl-ethanolamine (EE-GPME-DNP), whereas the faster component (8 %) consisted mainly of the corresponding 1-alkyl-2-acyl derivative (AE-GPME-DNP). These identifications are based on the following observations: 1) Synthetic samples of EE-GPME-DNP and AE-GPME-DNP were obtained by dinitrophenylating and meth-

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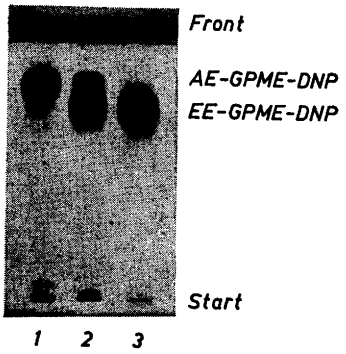


*Fig. 1.* TLC of dinitrophenylated and methylated egg GPE-lipids on silica gel G. Solvent: Ether-methanol (98:2, v/v). Detection: Charring with bisulfate solution.[11]

1. Fast fraction isolated by preparative TLC.
2. Original derivative of egg GPE-lipids.
3. Slow fraction isolated by preparative TLC.

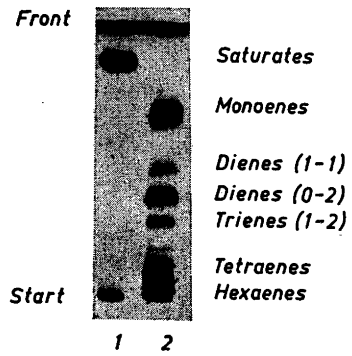


*Fig. 2.* Argentation TLC of dinitrophenylated and methylated phosphatidyl ethanolamines (EE-GPME-DNP). Solvent: Chloroform-methanol (98:2, v/v). Detection as in Fig. 1.

1. Methylated DNP-derivative of 1,2-dipalmitoyl-phosphatidyl-ethanolamine.
2. Methylated DNP-derivatives of egg phosphatidyl-ethanolamines.

ylating 1,2-dipalmitoyl-GPE (Fluka, AG, Buchs, Switzerland) and 1-palmityl-2-steroyl-GPE (a gift from Dr. T. H. Bevan, Bristol). The resulting model compounds behaved like the two egg lipids on TLC. 2) Also other unpolar forms of 1-alkyl-2-acyl-lipids migrate faster on silicic acid than corresponding diacyl lipids; this is true, *e.g.*, for diglyceride acetates [2] and dimethyl phosphatidates.[6] 3) The slower moving fraction of the egg lipid lost both of its two fatty chains under mild alkaline conditions,[8] whereas the fast moving fraction lost only one chain and was converted into a lyso-derivative. After remethylation with diazomethane this lyso-compound had similar mobility on TLC as dinitrophenylated and methylated lysophosphatidyl-ethanolamine. 4) Both fractions of the egg lipid were stable under mild acid conditions [8] known to cleave the vinyl ether linkages of the plasmalogens. 5) Carter *et al.*[9] have shown that egg phosphatidyl ethanolamines are accompanied by similar lipids containing the 1-alkyl grouping.

Both types of the methylated DNP-derivatives were resolved into several subfractions on silver nitrate containing silica gel G. In egg EE-GPME-DNP's argentation TLC revealed at least six distinct spots when chloroform-methanol (98:2, v/v) was used as solvent (Fig. 2). All these subfractions were isolated by preparative TLC, which was particularly easy with the yellow derivatives. Gas-liquid chromatography of the methyl esters of the component fatty acids (Table 1) showed that the most rapidly moving component (8.3 %) consisted of monoenoic EE-GPME-DNP's with one saturated and one monoenoic fatty acid, the second spot (2.4 %) contained dienes with two monounsaturated acids (type 1—1); the third spot (8.7 %) consisted of dienes with one saturated and one diunsaturated acid (type 0—2); the fourth subfraction (2.5 %) contained trienes of type 1—2; the fifth spot (37 %) contained mainly tetraenes of type 0—4, and the last fraction (40 %) hexaenes of type 0—6. Fig. 2 shows that also saturates and monoenes were separated quite well with chloroform-methanol (98:2, v/v). The polyenes with 4—6 double bonds were separated better by a mixture of chloroform-methanol-water (90:10:1, v/v/v).

The fatty acid composition of the hexaenoic subfraction is interesting. Table 1 suggests that in contrast to the other fractions the hexaenes might contain a large proportion of palmitic acid.

The AE-GPME-DNP's of egg gave on argentation TLC quite similar subfractions

*Table 1.* Fatty acid composition of EE-GPME-DNP-subfractions.

| Fraction | *Relative amounts of principal acids, %* | | | | | | |
|---|---|---|---|---|---|---|---|
| | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:4 | 22:6 |
| Monoenes | 13 | 35 | 52 | | | | |
| Dienes (1—1) | 2 | 6 | 82 | 5 | | | |
| Dienes (0—2) | 7 | 40 | 2 | 51 | | | |
| Trienes (1—2) | 5 | 5 | 35 | 47 | 7 | | |
| Tetraenes | 5 | 41 | 1 | | 3 | 49 | |
| Hexaenes | 24 | 26 | 3 | | | 3 | 44 |

as the EE-GPME-DNP's. This suggests that the diacyl and the alkyl-acyl forms of egg GPE-lipids consist of similar molecular populations. This is known to be the case with the different subtypes of ox-brain lecithins too.[11]

The silver-ion separations obtained with the methylated DNP-derivatives were based not only on the number, but also on the location of the unsaturated centers. The dienes of type 1—1 moved faster than the dienes of type 0—2; thus two double bonds which are in the middle of two adjacent chains (type 1—1) seem to interact with silver ions less strongly than two double bonds located in the same chain (type 0—2). The same observation has been recorded with triglycerides,[10] diglyceride acetates,[11] and dimethyl phosphatidates.[6] This probably means that the double bonds of the adjacent chains are close neighbours when the molecules are in their most stable conformations.

The remarkable degree of separation obtained by the present method is illustrated by the fact that comparable separations between the alkyl-acyl and the diacyl phosphatides, or between the different types of dienoic molecules, have not previously been reported with any native phospholipids. However, similar separations have recently been obtained in our laboratory with phosphatidic acid dimethyl esters.[6] It remains to be seen whether some modification of these approaches using "masked" polar groups can be applied to other types of phospho- and glycolipids.

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