## Discussion

Gas chromatography of the sphingolipid bases as their trimethylsilyl derivatives provides a simple and direct method for qualitative and quantitative estimations of the bases. Compared with the method described earlier (29), in which the aldehydes liberated by periodate were separated by gas chromatography, this procedure has obvious advantages. Since analyses are made of the bases themselves, several lengthy steps can be eliminated from the method. Furthermore, the first two carbon atoms of the bases are not lost to analysis, as they were in the periodate method, so that the new method can be used for separations of isotope-labeled bases and determinations of their specific activities at the same time. On a semipreparative scale, analytical columns can be used to obtain pure TMS derivatives, from which the free sphingolipid bases can be recovered easily and quantitatively by mild hydrolysis (9). A disadvantage of the present method is that bases are not well separated according to differences in olefinic bonds. Selective liquid phases such as nitrile silicone (XE-60) are not much better than SE-30 in this respect, though no investigation has been made of separations on polyester columns.

Methanolysis of sphingolipids under anhydrous acidic conditions leads to the formation of 3-*O*-methyl sphingosine in yields of 50% or more, as illustrated by this study. This compound and a related unknown base are formed from any *N*-acylated sphingosine, but not from the free base or from *N*-acyl dihydrosphingosine. The unknown by-product may be the 5-*O*-methyl-$\Delta^3$-sphingosine described recently by Weiss (33). The presence of *O*-methyl ethers complicates determinations of sphingolipid bases by TLC or gas chromatography and conditions were, therefore, sought which would completely eliminate their formation. Though we have not found such a reagent, the presence of water in the methanolysis mixture effectively inhibits the formation of *O*-methyl ethers to a large degree. Morrison and Smith (18) have described conditions for the quantitative liberation of sphingolipid bases and fatty acid methyl esters from sphingomyelin by methanolysis with boron trifluoride-methanol. We have not yet studied this condition with regard to the yield of *O*-methyl ethers; it may prove to be a superior reagent.

The presence of an unidentified sphingolipid base in sphingomyelin from human plasma presents an excellent example of the utilization of this method for structural work. The presence and concn of the compound have been demonstrated in this study. The base has been identified tentatively as $C_{16}$-sphingosine. Though it could probably never be separated from sphingosine by classical techniques, it will be possible to obtain small quantities of the pure base by preparative gas chromatography.

ACKNOWLEDGMENT

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# Individual Molecular Species of Different Phospholipid Classes. Part II. A Method of Analysis[1]

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## Abstract

New analytical possibilities arise when glycerophosphatides are converted into diglyceride acetates or analogous compounds: In this less polar form the phospholipids can be subjected to the usual methods of triglyceride fractionation, including chromatography on silica gel mixed with silver nitrate. This opens a route to subfractionation of various glycerophosphatide classes, and makes analysis of the individual molecular species potentially possible in many cases. The same approach can also be applied to the analysis of sphingomyelins.

Two methods are suitable for the conversion of glycerophosphatides into "diglyceride acetates": 1) Acetolysis in a mixture of acetic anhydride and acetic acid, and 2) treatment with phospholipase C (E.C. 3.1.4.3.) followed by acetylation. Acetolysis was used successfully with phosphatidyl choline, phosphatidyl ethanolamine and corresponding alkoxy phosphatide (native cephalin B), phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol, phosphatidic acid and cardiolipin. Phospholipase C from *Clostridium welchii* was used for native choline and ethanol-

[1] Part I. Proposal for a Method of Analysis. ACTA Chem. Scand. *18*, 271 (1964).

amine plasmalogens as well as for sphingomyelins. Although this enzyme does not attack pure ethanolamine phosphatides it did so in the presence of added (dipalmitoyl) phosphatidyl choline or serum sphingomyelin. The mixed substrates containing sphingomyelin proved particularly valuable, since the plasmalogenic diglyceride acetates were easily separated from the usual diglyceride acetates and ceramide diacetates on silicic acid.

The diglyceride acetates obtained from lecithins of hens' eggs, ox-brain and human serum were subjected to preparative TLC on Kieselgel G mixed with silver nitrate. The resulting subfractions were characterized by GLC analysis of the fatty acid methyl esters. When these data were combined with enzymic analysis of the acids occupying the alpha and beta positions of the molecules a rather detailed description of the lecithins became possible.

The use of diglyceride acetates provides a route to analysis of fatty acids in many phosphatidyl compounds which have been obtained previously only as mixtures with the alkoxy analogs.

## Introduction

The samples of different phospholipid classes as obtained from natural sources always contain many fatty acid components; hence they represent complicated mixtures of several molecular species. For instance a lecithin preparation containing only two fatty acids A and B could be composed of four different types of lecithin molecules; AA, AB, BA and BB. Quite obviously the true composition of any such phosphatide cannot be analyzed by simple fatty acid analysis, or even by analysis of the acids occupying the alpha and beta positions within the molecules; therefore, fractionation of the mixture of complex molecules themselves seems necessary. At first this seems a difficult task because of the great difference in the properties of the numerous phosphatide classes. However, in the present paper a simple and general method of fractionation is described that appears to be suitable for the analysis of the different molecular species within all known phosphatide classes. A preliminary report of this investigation has been published elsewhere (1).

The basic idea of this procedure is to convert the glycerophosphatides into diglyceride acetates and to subject the latter to the usual procedures of triglyceride fractionation. The conversion step serves two purposes: first, it makes the subsequent fractionation much easier, for such methods as GLC, reversed phase partition, and chromatography on adsorbents containing silver nitrate appear to be easily adapted to fractionation of diglyceride acetates, although use of such procedures seems difficult or impossible with the original polar phosphatides. Secondly, the conversion step helps to keep the number of necessary fractionation procedures to a minimum. Since all types of phosphatidyl lipids can be converted into diglyceride acetates, a procedure for their fractionation is all that is required in the analysis of all the phosphatides. Without the conversion, on the other hand, the fractionation of lecithins would represent one problem, that of phosphatidyl ethanolamines another problem and so on.

## Conversion of Phosphatidyl Lipids Into Diglyceride Acetates

Two different methods for converting glycerophosphatides into diglyceride acetates have been used; acetolysis under conditions similar to those described by Malkin and co-workers (2), and enzymic cleavage of phosphatidyl lipids into diglycerides with phospholipase C (phosphatidylcholine choline phosphohydrolase; EC 3.1.4.3.) (3) and subsequent acetylation with pyridine and acetic anhydride.

For the acetolysis the phosphatides (0.6–60 μmoles) and the solvent, 2 ml of acetic anhydride-acetic acid (2:3) (4) are sealed in ampules and heated in an oven at 145C. The reaction mixture is evaporated to dryness and the dried residue partitioned in the system chloroform-methanol-water (8:4:3) (5); The phosphorus content (6) of the aqueous methanolic layer gives the amount of the acetolyzed phospholipids. Figure 1 shows the observed rate of acetolysis for some glycerophospholipids. It is of some interest that phosphatidylethanolamine (and phosphatidyl serine) were converted to the acetates faster than phosphatidyl choline. The deacylated alpha-alkoxy derivative of glycerylphosphorylcholine (7) reacted more slowly than the acylated alkoxy derivative or the usual lecithin.

Our study of the lipophilic acetolysis products was begun with a careful analysis of the reaction mixture obtained from a highly purified specimen of egg lecithin. After 4 hours' heating with the acetolysis mixture the sample yielded a lipophilic fraction, which on TLC showed, besides unchanged lecithin, a major constituent of the same mobility as acetylated 1,2-diglycerides (Fig. 2). The diglyceride acetates are seen to be easily separated from the usual triglycerides, and also from diacetates of the common monoglycerides. After 48 hours' heating with 2 ml of the acetolysis mixture another sample (66.7 μmoles P) of the lecithin gave a crude lipophilic fraction which contained 65.6 μmoles glycerol (8) and 202 μmoles carboxylic ester (9) but less than 0.1 μmoles phosphorus. The reaction product most probably consisted of rather pure diglyceride acetates since the molar ester-glycerol ratio was 3.06:1.00 and one third of the ester linkages was contributed by acetate group (Table I). Furthermore, the yield of the reaction was seen to be excellent in that 98% of the original glycerol, but only 0.2% phosphorus was recovered in the lipid fraction.

It does not seem probable that the acetic acid would add to the double bonds, since this would be reflected, e.g., in higher total ester to glycerol ratios than 3:1. However, the problem of possible fatty acid exchange between neighboring phosphatide or diglyceride molecules required special investigation, since such exchange would make subsequent analysis of the diglyceride acetates pointless. Therefore 2 mg samples of diolein and dipalmitin were subjected together to the conditions of acetolysis, the reaction volume being 1.0 ml. Had any exchange of acids occurred the reaction

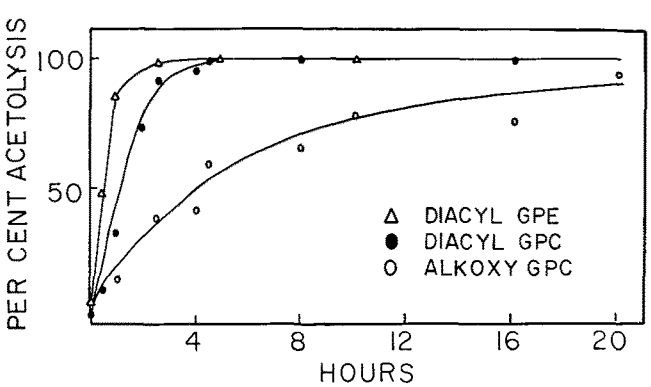


FIG. 1. Time course of acetolysis.

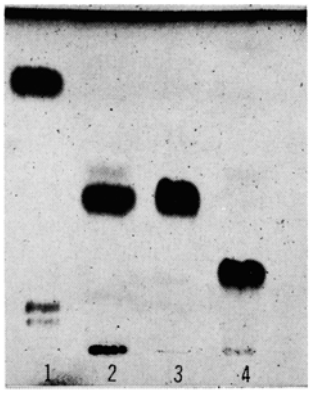


FIG. 2. TLC of different types of triglycerides on Kieselgel G. Solvent: petroleum ether-diethylether (4:1): *1*: Triolein. *2*: Acetolysis product of egg lecithin. *3*: Acetylated 1,2-diglyceride. *4*: Mono-olein diacetate. The plate was charred with sulfuric acid.

mixture would have contained monoenoic diglyceride acetates in addition to the saturated and dienoic ones.* However when the reaction mixture was analyzed on a silver nitrate Kiesel-gel G plate, no monoenoic compounds were detected (Fig. 3). A similar experiment with the same result was carried out also with 1 mg samples of dipalmitoyl-phosphatidyl ethanolamine and dioleoyl-phosphatidyl ethanolamine. Thus it appears that acetolysis of glycerophosphatides can be carried out without any appreciable intermolecular exchange of fatty acids.

One minor side reaction, however, takes place during the acetolysis; the reaction mixtures obtained from egg lecithin invariably seemed to contain small amounts of 1,3-diglyceride acetates in addition to the expected, and quite predominating, 1,2-diglycerides. This is shown on TLC (Fig. 4) where the two types of acetates are separated quite as the corresponding diglycerides themselves (11). The separation of these two types of isomers is possible even on preparative scale TLC. However, this will probably not often be necessary since in principle the intramolecular movements do not hinder the analysis of the fatty acid combinations.

Cis-trans isomerization was not observed, but potential trouble is caused by oxidative reactions. In preparative scale experiments with lipids of high content of polyenoic acids some darkening of the reaction mixtures was inevitable. It could not be prevented by use of tert. butyl-p-hydroxy-anisol as antioxidant nor by the presence of nitrogen and the absence of light. Samples isolated immediately before the acetolysis might not darken. The samples used in this study were not quite fresh. However, even when there was some darkening of the reaction mixtures, quite colorless material could easily be isolated in satisfactory yields

TABLE I

Analysis of Acetyl Groups in Glycerides

| Lipid | Total ester | Long-chain ester[a] | Acetate ester[b] | Molar ratio total to long-chain ester |
|---|---|---|---|---|
| | μmoles | μmoles | μmoles | |
| Triolein | 0.516 | 0.517 | 0.000 | 3.00:3.00 |
| Diolein acetate | 0.782 | 0.525 | 0.257 | 3.00:2.02 |
| Dipalmitin acetate | 0.656 | 0.432 | 0.224 | 3.00:1.96 |
| Mono-olein diacetate | 0.507 | 0.184 | 0.323 | 3.00:1.09 |
| Triacetin | 0.406 | 0.016 | 0.390 | 3.00:0.12 |
| Diglyceride acetate form egg lecithin | 0.960 | 0.636 | 0.324 | 3.00:1.98 |

[a] Measured as follows: The lipid was subjected to methanolysis at 20C in 0.05 N NaOH in moist chloroform-methanol (3:4) (10). Partition of the reaction mixture in chloroform-methanol-water (8:4:3) gave the methylesters in chloroform solution. Evaporation and drying in high vacuum at 20C for 5 min eliminated methyl acetate, and the remaining long-chain compounds were assayed for carboxyl ester.

[b] Calculated as the difference between total and long-chain ester; other short chain esters would naturally behave more or less like methyl acetate.

* A dienoic phospholipid or diglyceride is defined as a lipid containing two double bonds which may or may not be in the same fatty acid.

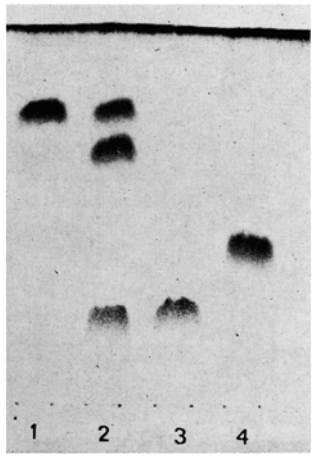


FIG. 3. TLC of diglyceride acetates on a "silver plate." Solvent: benzene-chloroform (9:1). *1*: 1,3-Dipalmitin acetate. *2*: Acetolysis product of a mixture of 1,3-dipalmitin and 1,3-diolein (the second spot is 1,2-dipalmitin acetate). *3*: 1,3-diolein acetate. *4*: A monoenoic 1,2-diglyceride acetate.

after preparative TLC on Kieselgel G in petroleum ether-diethylether (4:1). The dark material was retained much more strongly than the diglyceride acetates.

Besides egg lecithin, brain and serum lecithins also have given good yield of diglyceride acetates upon acetolysis, and the same is true of phosphatidic acid, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol and phosphatidyl glycerol. Even cardiolipin gave diglyceride acetates under these conditions.

The enzymic method used to convert the phosphatidyl lipids into diglyceride acetates was an adaptation of a procedure described by Sribney and Kennedy (12). It can be illustrated by an example: A sample of 96.1 μmoles of highly purified lecithin from human serum (13) was suspended into 15 ml water and 7 ml of a buffered enzyme solution, pH 7.3, were added. [3 mmoles tris (hydroxymethyl) aminomethane, 0.6 mmoles $CaCl_2$ and 2.5 mg phospholipase C (alpha-toxin of *Cl. welchii*) obtained from Sigma Chemical Company, St. Louis, Mo.] Finally, 20 ml diethyl ether was added and the mixture stirred. The reaction was followed by TLC analysis. After 3 hr, when the reaction seemed complete, the ether layer was separated and the water layer was reextracted three times with 30 ml of ether. The combined ether extracts contained only 2 μmoles phosphorus and showed a rather pure 1,2-diglyceride spot on TLC. The crude product was acetylated with 0.5 ml pyridine and 2.5 ml acetic anhydride by keeping the reaction mixture for 2 hr at 70C and then over night at 20C. Evaporation and partition in 60 ml of chloroform-methanol-water (8:4:3) gave 60.5 mg of 1,2-diglyceride acetates which appeared rather pure on TLC and contained 287 μmoles carboxylic ester. Thus the yield was over 99%.

## Analysis of Simple Lecithins

Chromatography on adsorbents containing silver

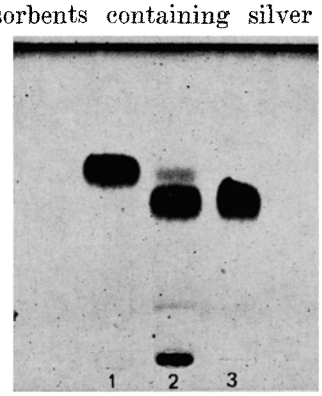


FIG. 4. TLC of diglyceride acetates on Kieselgel G. Solvent: petroleum, ether-diethylether (4:1). *1*: 1,3-diolein acetate. *2*: Acetolysis product of egg lecithin. *3*: 1,2-diglyceride acetates.

nitrate has been used for the fractionation of the diglyceride acetate mixtures obtained from simple lecithins (14). This type of fractionation separates the diglyceride acetates according to the total number (and geometry) of double bonds in the molecules; fully saturated molecules travel faster than monoenes, and these precede dienes and so on.

We have used both column chromatography and preparative TLC for these separations, and prefer the latter. The following example demonstrates the details of our present procedure. A sample (51.3 $\mu$moles) of chromatographically purified diglyceride acetates derived from egg lecithin was dissolved in 300 $\mu$l chloroform and applied to 6 silver nitrate Kiselgel G plates (200 × 200 × 0.25 mm). The adsorbent was prepared by mixing in the usual way 3 parts $AgNO_3$ and 10 parts Kieselgel G in 20 parts water. After development in benzene-chloroform (9:1) the plates were sprayed with Rhodamine 6G and inspected under ultraviolet light. Four yellow zones were visible, and corresponded to fully saturated, monoenoic, dienoic and polyenoic diglyceride acetates. The individual zones were scraped off and extracted by elution with chloroform-methanol (9:1) in chromatographic columns. The eluates were washed with one-fifth volume of water and finally chromatographed on small silicic acid columns for removal of Rhodamine 6G. In more recent work the separation between diglyceride acetates and the dye has been carried out by preparative TLC. The yields of the resulting purified subfractions were obtained by ester analysis. Good recoveries were obtained (94.7% and 97.8%) as shown in Table II.

The purified subfractions were colorless and showed unchanged diglyceride acetate spots on TLC. The saturated fraction, however, was contaminated with Rhodamine 6G components. Further characterization of the purified subfractions was obtained on silver nitrate Kiselgel G plates with dipalmitin acetate and diolein acetate as reference compounds. Figure 5 shows that the second, third and fourth zone materials were really pure and well separated from each other. It is also evident that the material from the second zone must be monoenoic since it occupies a position between the saturated and dienoic reference compounds. The diglyceride acetates from the third zone must be dienoic as they run approximately to the same position as diolein acetate. The material from the fourth zone finally must contain polyenes, because it runs so much slower than the dienes. The impure material of the first zone is not shown in Figure 5, but since it runs ahead of the monoenes with about the same mobility as dipalmitin acetate it was considered to be saturated in nature.

Next the component fatty acids were analyzed; The diglycerides were methanolyzed in dry methanol-sulfuric acid 94/6 (v:v) and the methylesters analyzed by GLC. Figure 6 shows the tracing obtained from the acids of the original diglyceride acetates. The picture was identical with that obtained from the parent lecithins. The fatty acid spectra of the monoenoic, dienoic and polyenoic diglyceride acetates are also given in Figure 6.

TABLE II
Subfractions of Diglyceride Acetates Derived from Egg Lecithins

| Subfractions | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|
| | $\mu$moles | % | $\mu$moles | % |
| Saturates | 1.68 | 3.3 | 1.07 | 2.1 |
| *Monoenes* | 32.2 | 62.8 | 31.5 | 61.5 |
| Dienes | 11.3 | 22.0 | 12.1 | 23.6 |
| Polyenes | 4.97 | 9.7 | 3.83 | 7.5 |
| Total | 50.1 | 97.8 | 48.5 | 94.7 |

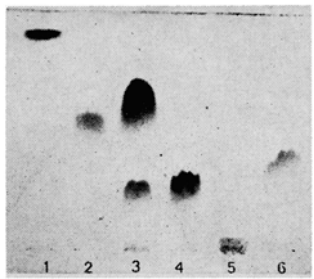


FIG. 5. TLC of diglyceride acetates on a "silver plate." Solvent: benzene-chloroform (9:1). *1*: Dipalmitin acetate. *2*: Material from the second zone of prep. TLC. *3*: Diglyceride acetates from egg lecithins, starting material for prep. TLC. *4*: Material from the third zone of prep. TLC. *5*: Material from the fourth zone of prep. TLC. *6*: Diolein acetate.

The monoenoic diglyceride acetates indeed appeared to contain saturated and monoenoic acids in equimolar amounts whereas the more unsaturated acids were lacking. A separate experiment with phospholipase A (15) showed that the parent lecithins contained almost exclusively myristic, palmitic and stearic acid in the alpha position whereas all the unsaturated acid and a little palmitic acid were found in the beta position of the molecules. This information together with the result of fatty acid analysis settles the composition of the monoenoic fraction: Only three groups of molecules seemed to be present, namely 1-myristoyl-2-monounsaturated acyl lecithins (trace), 1-palmitoyl-2-monounsaturated acyl lecithins (74%) and 1-stearoyl-

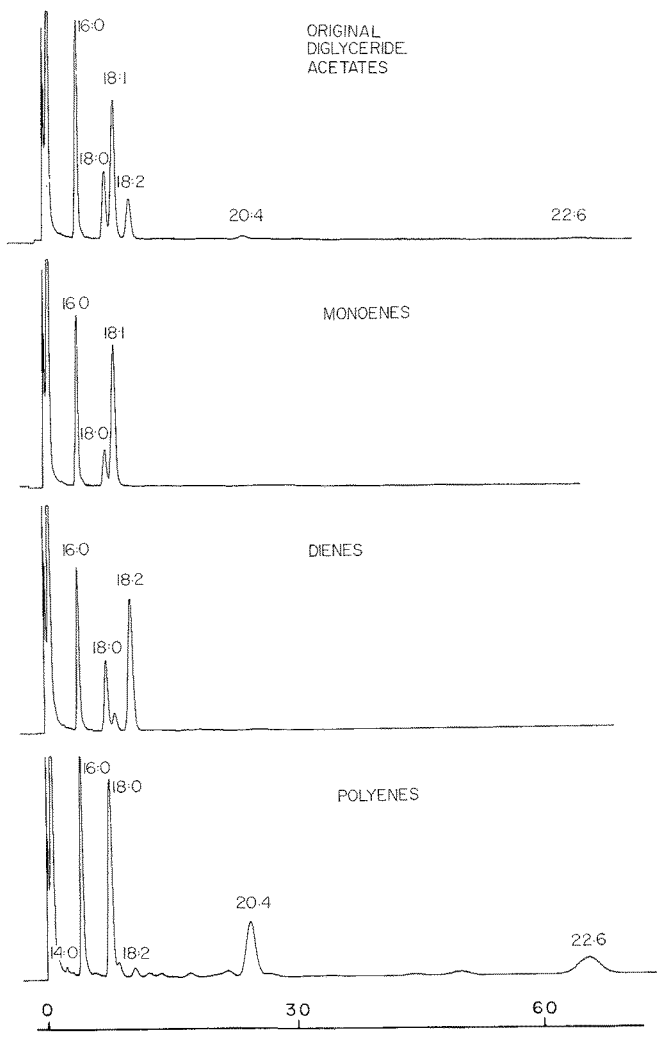


FIG. 6. GLC of methylesters of egg lecithin subfractions. The experimental conditions were as follows: A Barber-Colman apparatus; Argon detector with radium as source of ionizing radiation; detector voltage 820; a 6 ft glass column of EGSS-X (Applied Science, State College, Pa.) operated at 182C.

2-monounsaturated acyl lecithins (24%). In each of the three groups the monounsaturated component was either a hexadecenoic or an octadecenoic acid but it is not known whether both occurred in all three groups. Further separation of the monoenoic fraction, e.g., with GLC, will very likely answer this question in the near future.

Similar analysis of the dienoic fraction suggested the following composition: 1-myristoyl-2-octadecadienoic acyl lecithins (trace), 1-palmitoyl-2-octadecadienoic acyl lecithins (64%), 1-stearyl-2-octadecadienoic acyl lecithins (36%). However GLC analysis suggests that a small amount of dioleoyl type of lecithins might also have been present.

The polyenes quite expectedly showed approximately equimolar amount of saturated and polyenoic acids, but as this fraction appears much more complex than the previous ones, further fractionation with silver nitrate adsorption chromatography and then with partition methods is necessary before its composition can be given. However an interesting feature is already evident in Figure 6: The polyenoic fraction seemed to contain a higher relative proportion of stearic acid than the monoenes and dienes.

Lecithins of ox-brain and human serum, which are also examples of relatively pure phosphatidylcholines were quantitatively isolated in a very pure form and subjected to similar analysis as described above. Brain

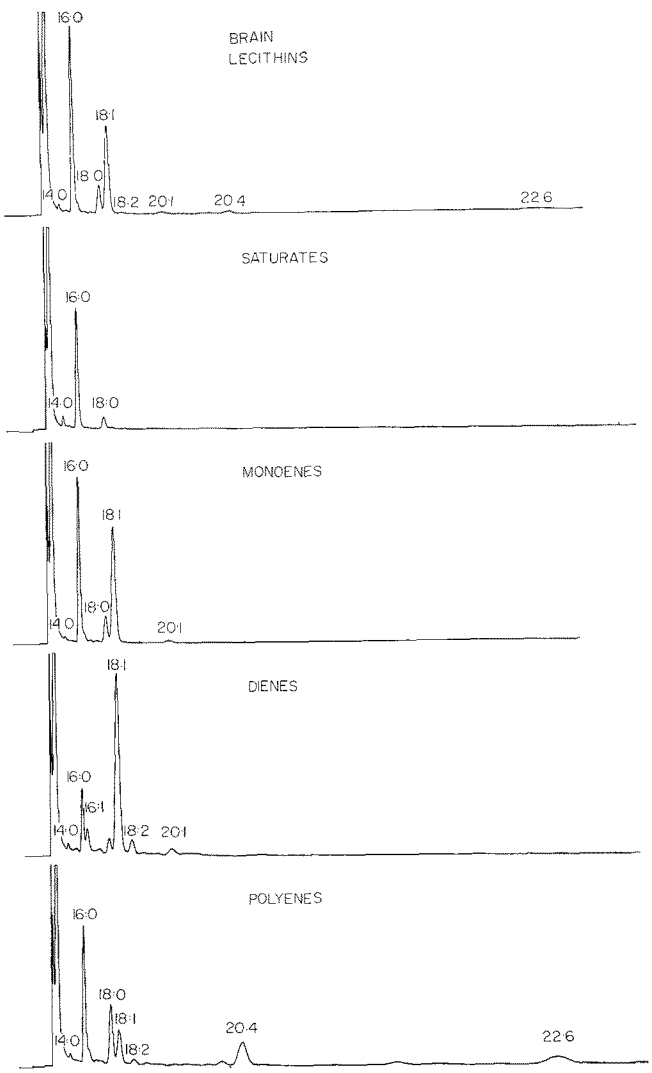


FIG. 7. GLC of methylesters of brain lecithin subfractions. Conditions as in Fig. 6.

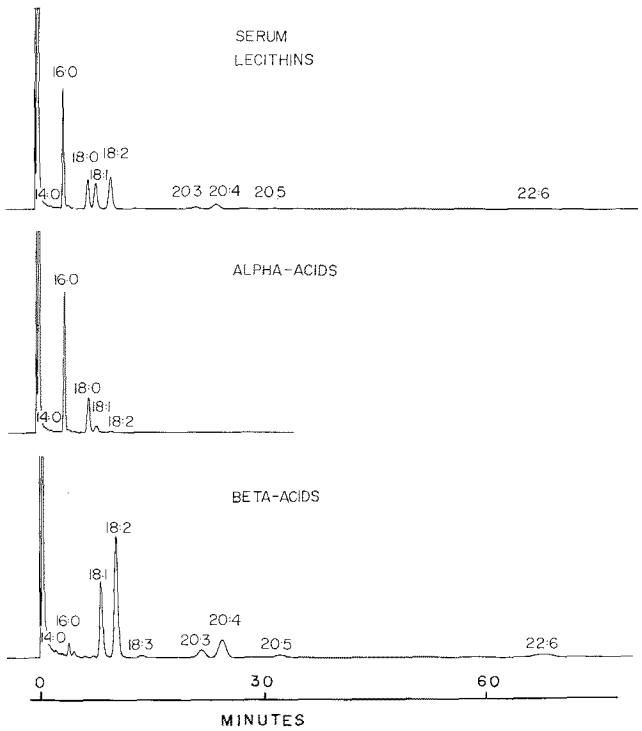


FIG. 8. GLC of methylesters of human serum lecithins. Conditions as in Fig. 6.

lecithins gave pure diglyceride acetates in a quantitative yield, and a sample of 103 μmoles of these was fractionated on twenty silver nitrate Kieselgel G plates. After purification of the different subfractions the following yields were obtained: saturates 14.8 μmoles, monenes 50.7 μmoles, dienes 5.8 μmoles, and polyenes 11.5 μmoles (an overall recovery of 80%). The GLC record of the corresponding methyl esters is given in Figure 7. The high content of totally saturated lecithins is striking when compared to egg lecithins. Another difference is that the dienoic fraction here consists principally of the dioleyl type of lecithin and less of the palmitoyl-linoleoyl type molecules found in egg lecithins. The very complex polyenoic fraction was subjected to refractionation with chloroform-methanol (97:3) on plates containing silver nitrate. In this system the monoenes and dienes moved with $R_f$ values of 0.93 and 0.73, respectively. The polyenes appeared to be separated into four zones in a preparative experiment. Palmitate, stearate, and oleate were the alpha acids found in each of these zones; linoleate was in the most rapid zone, linolenate in the next, arachidonate in the third, and dodecahexaenoate in the last. Interestingly, stearic acid constituted a rather high proportion of the saturated acid which appeared together with arachidonic acid.

The lecithins of human serum appeared the most complex. Their fatty acid pattern as well as the records of their alpha and beta acids are given in Figure 8. The fractionation of the corresponding diglyceride acetates gave 2% saturates, 21% monoenes, 36% dienes, and 40% polyenes. The GLC records of these methyl esters is shown in Figure 9.

## Analysis of Samples Containing Plasmalogens and Alkoxy Phosphatides

The natural phosphatidyl lipids often occur together with corresponding plasmalogens and acylated alkoxy phosphatides. Such mixtures present a more complex problem than the simple lecithins described above.

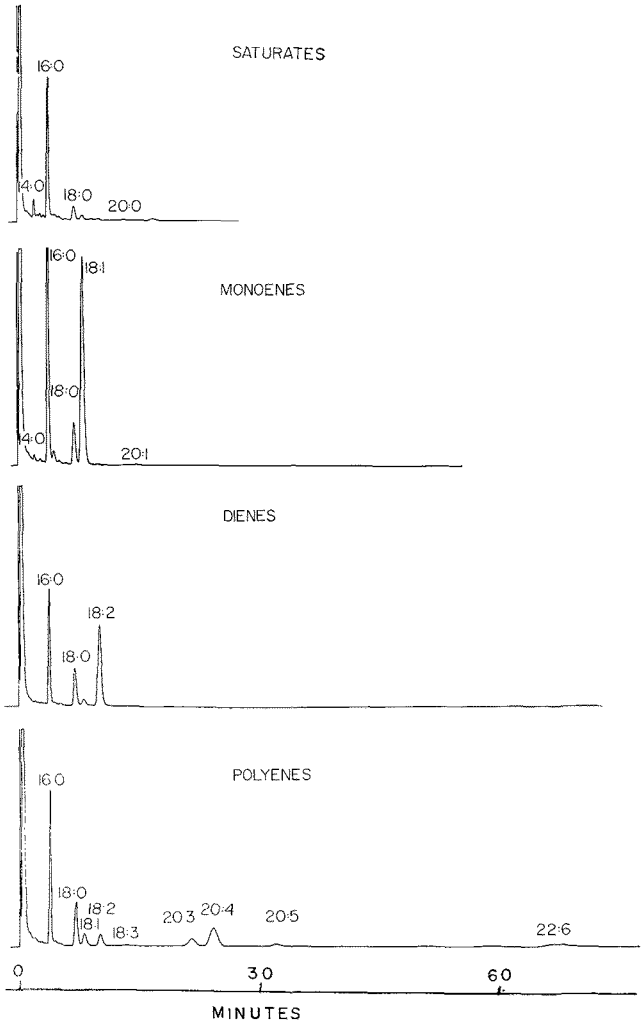


FIG. 9. GLC of methylesters of serum lecithin subfractions. Conditions as in Fig. 6.

These three subtypes of glycerophosphatide classes cannot at present be separated by chromatographic means, but some methods of selective hydrolysis are available for their partial fractionation. Acid hydrolysis (16) destroys the plasmalogens and yields a mixture of the phosphatidyl lipids and acylated alkoxy phosphatides. Alkaline hydrolysis (17) can be used for selective deacylation of the phosphatidyl lipids whereby mixtures of native plasmalogens and acylated alkoxy phosphatides are obtained. Finally a combination of these two hydrolytic procedures can be used (13) for partial isolation of the pure acylated alkoxy phosphatides.

The dephosphorylation reactions described above seem useful in two ways in the analysis of these three subtypes: First, in nonpolar form they can be fractionated by chromatographic means quite successfully. Figure 10 shows that on TLC the "diglyceride acetates" derived from plasmalogens and acylated alkoxy phosphatides run more rapidly than those derived from the phosphatidyl lipids. This type of separation provides a route to uncontaminated phosphatidyl lipids; previously these compounds have not been separated from the accompanying alkoxy phosphatides. Secondly, the "diglyceride acetates" derived from the plasmalogens and alkoxy phosphatides are quite suitable for fractionation on silver nitrate adsorbents just as the usual diglyceride acetates.

There appear to be no difficulties in the dephosphorylation of the acylated alkoxy phosphatides. Both

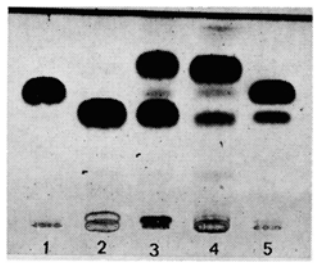


FIG. 10. TLC of different types of diglyceride acetates on Kieselgel G. Solvent: petroleum ether-diethylether (4:1). *1*: 1,3-diolein acetate. *2*: crude 1,2-diglyceride-acetate. *3*: Enzymic hydrolysate of ox-brain GPE lipids after acetylation; the upper spot is a mixture of plasmalogenic diglyceride acetates and corresponding alkoxy analogs. *4*: enzymic hydrolysate of an "isolated" sample of choline plasmalogens and alkoxy derivatives after acetylation. *5*: a mixture of 1,3-dipalmitin acetate and 1,2-dipalmitin acetate.

acetolysis and enzymic cleavage with subsequent acetylation can be used to yield glycerolethers containing one long-chain acyl- and one acetyl-group. In the case of plasmalogens, however, the conditions of acetolysis lead to some destruction of the vinyl ether linkages and the enzymic conversion method must be used for samples containing plasmalogen.

The conditions described above must be modified in analyzing ethanolamine phosphatides since the enzyme of *Cl. welchii* does not attack these lipids in pure form (18). *Bacillus cereus* is known to contain an enzyme which will attack even negatively changed phosphatide classes (19), but in this investigation advantage was taken of the fact even the enzyme of *Cl. welchii* will catalyze the hydrolysis of phosphatidyl ethanolamine in the presence of lecithin (20). Therefore in the enzymatic hydrolysis of glycerylphosphorylethanolamine lipids, phosphatidyl choline was added to the mixture. As expected the glycerylphosphorylethanolamine lipids were readily attacked in the mixture although they remained quite intact if lecithin was not added. The hydrolysate contained ordinary diglycerides formed both from the lecithins and the phosphatidyl ethanolamines but the "plasmalogenic diglycerides" and the alkoxy analogs originated from the glycerylphosphorylethanolamine-preparation only, since the added lecithin sample was a synthetic specimen of pure diacyl form. Subsequent acetylation of the crude lipids of the enzymic hydrolysate, and isolation of the "plasmalogenic diglyceride acetate" fraction therefore solved the problem quite satisfactorily.

The experimental details were as follows: A representative sample of highly purified ox-brain glycerylphosphorylethanolamine-lipids (14.3 mg P) was suspended in 130 ml water and 70 ml buffered enzyme solution, pH 7.3, containing 25 mg phospholipase C, 6 mmoles $CaCl_2$ and 30 mmoles tris (hydroxymethyl) aminomethane were added; 100 ml of diethyl ether were finally added and continuous stirring was applied. The reaction was followed by TLC in two systems; The first was suitable for the detection of diglycerides, and the second was devised for the original glycerylphosphorylethanolamine-lipids. After 20 hours' reaction time no reaction had occurred. Then 25 mg fresh enzyme and 300 mg DL-dipalmitoyllecithin (Fluka AG., Switzerland) were added and the stirring continued: After two hours "plasmalogenic diglycerides" as well as unsaturated and saturated diglycerides could be observed on TLC plates. After additional 20 hours' stirring the reaction mixture was thoroughly extracted with ether, and the crude product (530 mg total weight, 5.42 mg P) was chromatographed on a column packed with 70 g of Mallinckrodts' silicic acid. The fractions eluted with

chloroform were free of phosphorus and contained only the diglycerides and the corresponding plasmalogenic and alkoxy analogs. A preparation was pooled from these fractions which contained all the "plasmalogenic diglycerides" and the "alkoxy diglycerides" together with considerable amounts of ordinary diglycerides. Some fractionation between the different "diglyceride" subtypes had occurred on the column so that the pooled sample did not contain all the ordinary diglycerides present. A part of this pooled preparation was acetylated and a sample (41.5 mg) of the crude acetates was fractionated on 13 Kieselgel G plates (200 × 200 × 0.25 mm) with petroleum ether-diethylether (4:1) as solvent. (Larger loads have been used subsequently; for instance a sample of 154 mg has been separated successfully on six plates of 1 mm thickness.) The plates were sprayed with Rhodamine 6G and the two principal zones were scraped off and extracted by elution with chloroform-methanol (1:1) and pure chloroform in chromatographic columns. The zone containing the "plasmalogenic diglyceride acetates" and the alkoxy analogs gave 17.0 mg lipids, whereas the yield of the ordinary diglyceride acetates was 24.7 mg. After elimination of the dye, both preparations were pure according to analytical TLC plates (Fig. 11).

The addition of sphingomyelin was also used to increase the rate of hydrolysis of glycerylphosphorylethanolamine lipids by the *Cl. welchii* system. Since sphingomyelin did not contribute any diglycerides to the hydrolysate a complete analysis of the glycerylphosphorylethanolamine lipids was possible. An example of this type of experiment included the following: 312 mg ox brain glycerylphosphorylethanolamine lipids (22.66 mg P were mixed with 876 mg human serum sphingomyelins (28.64 mg P) and treated with phospholipase C from *Cl. welchii* as described above. After 23 hr when TLC analysis showed that the reaction was complete, the mixture was extracted and the crude lipids (945 mg) were chromatographed on a column containing 70 g of silicic acid. Elution with chloroform gave 124 mg pure "plasmalogenic" and alkoxy diglycerides, 120 mg of almost pure ordinary diglycerides, and chloroform-methanol (9:1) yielded 651 mg pure ceramides. The separation of the middle fraction was then completed by preparative TLC of the acetates.

The "plasmalogenic diglyceride acetates" were thus obtained together with the "alkoxy diglyceride acetates" from the two experiments. A sample (154 mg) of this mixture was separated on six silver nitrate-silicagel G plates (200 × 200 × 1 mm) with benzene-chloroform (9:1) as solvent. Four principal zones were again obtained, and although we have not yet carried out any GLC-analyses we believe that these represent saturates (4.5 mg after separation of the dye), monoenes (25 mg), dienes (33 mg) and polyenes (74 mg).

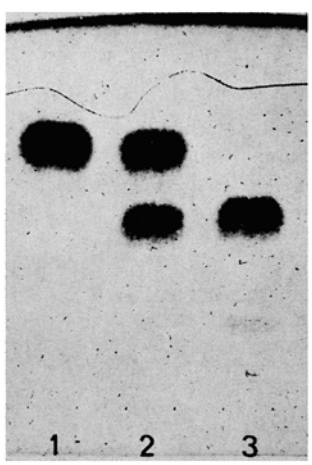


FIG. 11. TLC of different types of diglyceride acetates on Kieselgel G; Solvent: Petroleum ether-diethylether (4:1). 1: Plasmalogenic diglyceride acetates (together with alkoxy analogs) after prep. TLC. 2: Mixtures of 1 [illegible] 3 before prep. TLC. 3: [illegible]inary diglyceride acetates [illegible]er prep. TLC.

## Analysis of Sphingomyelins

The great potential value of the fractionation of complex lipids in nonpolar form is demonstrated also by sphingomyelins: For instance, the sphingomyelins of human serum are known to contain several "sphingosine bases" (21) and many different fatty acids (22) but nothing is known about the actual acid-base combinations in this or other sphingomyelin samples. However, when the sphingomyelins were dephosphorylated with phospholipase C and the resulting "ceramides" acetylated, preparations of "ceramide diacetates" were obtained which could be separated into several subfractions in preliminary experiments with TLC.

A sample (146 μmoles) of ceramide diacetates derived from serum sphingomyelins was applied to 22 silver nitrate Kieselgel C plates (200 × 200 × 0.25 mm) and chromatographed with chloroform-methanol (98:2). This gave two strong lipid zones besides a few, more faint and slower moving zones. All these materials were recovered, separated from the dye on small columns and analyzed. TLC showed that the different zones probably all were ceramide diacetates. GLC of the methyl esters proved interesting: Only saturated acids ($C_{14}$–$C_{24}$) were present in the first zone (73 μmoles) whereas "nervonic" acid predominated in the second zone (28 μmoles). The slower moving materials (18 μmoles) in turn showed both the saturated as well the monoenoic acids. This probably means that the slow material contained a more unsaturated base than the principal components. This experiment shows just one more example of the versatility which makes the dephosphorylation method different from other procedures of phospholipid subfractionation (23–27).

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