• TECHNICAL

Quantitative Gas Chromatography in the Structural Characterization of Glyceryl Phosphatides[1]

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Abstract

Inclusion of gas chromatography of diglycerides in the various schemes proposed for subfractionation of natural glyceryl phosphatides increases the accuracy of identification and quantitation of the individual molecular species. This is due to its efficiency in classifying the molecular weights and proportions of the diglycerides recovered from thin-layer chromatography according to their degree of unsaturation. Determination of the complete structure of glyceryl phosphatides requires standardization of all steps of the analytical system including lipid extractions, enzyme hydrolyses, and thin-layer and gas chromatography.

This presentation reviews some of the practical aspects of quantitative gas chromatography of diglycerides and fatty acids as applied in the determination of the molecular species of glyceryl phosphatides. It is shown that a good gas chromatographic technique effectively counteracts weaknesses in other analytical steps. The additional cross-checks provided by the gas chromatography of the diglycerides greatly improve the overall accuracy of the data and frequently permit perfect reconstitution of the overall and positional distribution of fatty acids, which must be the ultimate test of the success of the entire analytical scheme.

Introduction

THERE IS EVIDENCE that different species of lecithins extracted from a given tissue show significant variations in metabolic turnover (1–3). Although this could be a reflection of different rates of turnover of separate lecithin pools within a tissue, it has been concluded (1–3) that such variations in the dynamic state of the various lecithin molecules are due to differences in their chemical structure. It may therefore be rewarding to approach the study of the lecithins and other glyceryl phosphatides of membranes and lipoproteins as individual molecules rather than as general classes of phospholipids.

The analysis of glyceryl phosphatides in terms of molecular species has been accomplished (4) by chromatographic techniques using silica gel impregnated with silver nitrate. Although a separation of intact phospholipids can be achieved by a modification of Arvidson's method (3), most investigators have converted the phospholipids into less polar compounds before chromatography. This fractionation depends on the total number of double bonds in the diglyceride moiety and remains relatively unaffected by the chain length of the fatty acids or the molecular weight of the diglyceride. Exact pairings of the constituent fatty acids are therefore possible only for the major diglycerides or for very simple diglyceride mixtures, where the fatty acid data alone suffice. Addition of gas chromatographic analysis of the diglycerides, on the basis of carbon number, corrects this deficiency and provides experimental measurements adequate for exact assignment of fatty acid associations for most diglycerides (5).

It is the purpose of this paper to review those aspects of quantitative gas chromatography of diglycerides and fatty acids which are pertinent to the structural characterization of glyceryl phosphatides. The review is preceded by a brief description of the general scheme of the analysis and the methods of lecithin isolation, conversion into diglycerides, and their resolution on silica gel in the presence of silver nitrate.

General Scheme of Analysis

Fig. 1 represents the structures of four of the most common glyceryl phosphatides. To this group may be added phosphatidyl glycerol, cardiolipin and any other complex lipids containing diglyceride moieties. These natural phospholipid classes contain several different fatty acids each, and represent complex mixtures of various molecular species. Although a specific positional placement of the fatty acids within the molecules of some of the phospholipids has been recognized (6), detailed studies of the molecular species of phospholipids have only recently become feasible.

The most effective techniques of subfractionation of the glyceryl phosphatides within a chemical class are based upon the methods originally developed for the analysis of mixtures of natural glycerides. These procedures involve an initial dephosphorylation step, which is followed by TLC and GLC separation of the diglycerides and the identification of the component fatty acids. The diglycerides or diglyceride acetates, however, must be prepared by hydrolysis with specific phospholipases. Direct conversion of phospholipids into the diglyceride acetates by acetolysis has been shown (7,8) to result in a partial migration of the

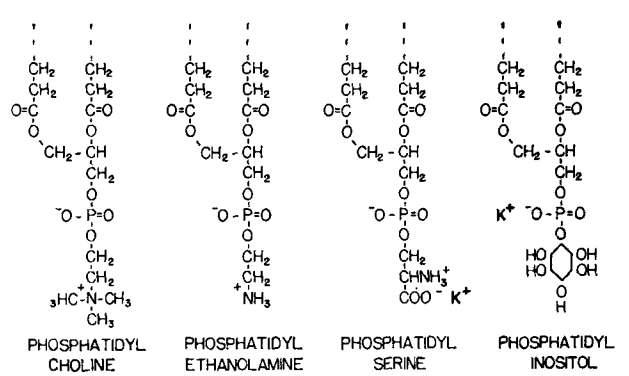

FIG. 1. Partial structures of four of the most common glyceryl phosphatides.

[1] Presented at the Short Course on Quantitative Gas-Liquid Chromatography conducted by the AOCS at Rice University, Houston, Texas, July 30–Aug. 4, 1967.

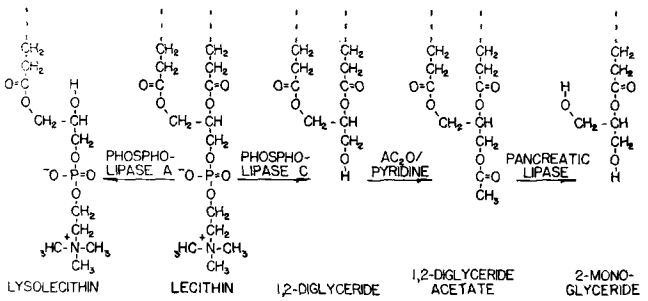

FIG. 2. Summary of the basic enzymatic and chemical steps used in the determination of the molecular species of glyceryl phosphatides.

acyl groups. While free diglycerides have given satisfactory resolutions on both silica gel and gas chromatography, isomerization is a potential hazard which can be avoided by using the diglyceride acetates for chromatography.

There may be, however, a need for a method retaining the phosphorus during the preliminary fractionation, to allow study of p^{32} incorporation. This can be accomplished by a preliminary segregation of the intact phosphatides by argentation TLC or of the dimethyl esters of the derived phosphatidic acids (9) by reverse phase TLC.

Fig. 2 summarizes the basic enzymatic and chemical steps involved in the analyses, which are performed either on the total lecithin mixture or on appropriate subfractions thereof. The actual sequence of steps taken in these transformations depends on the particular glyceryl phosphatide at hand and the personal preference of the investigator. In all cases, however, it is essential that the starting materials are representative of the total phosphatide class of the tissue and that any subfractions are derived in a systematic and representative manner. Nonspecific contamination from solvents and containers is a constant danger as is autoxidation and selective loss of the polyunsaturated components on adsorption chromatography.

Preparation of Samples

Pure solvents, clean containers and rapid processing of samples were the best insurance for obtaining good reconstitution of the analytical data. Prior to use, all solvents and reagents were tested for lipid contaminants by evaporating appropriate quantities of the solvent, or a chloroform extract of the reagent, to dryness, and injecting a chloroform solution of the residue into the gas chromatograph under the working conditions (following transmethylation, if necessary). All enzyme preparations were autodigested for about 30 min under the incubation conditions and the digests exhaustively extracted with diethyl ether prior to the addition of the substrate. Storage of lipid extracts was limited to less than one week at −20C in a petroleum or chloroform solution. No antioxidants were used.

Isolation of Glyceryl Phosphatides

This involves the preparation of a total lipid extract of the tissue in chloroform-methanol (2:1, v/v) using the method of Bligh and Dyer (10). The phases are separated by centrifugation and the chloroform layer evaporated to dryness. The desired phospholipid class may then be isolated by adsorption chromatography using columns (11) or plates (12) of silica gel and appropriate solvent systems. The lipid fractions obtained by the preparative techniques

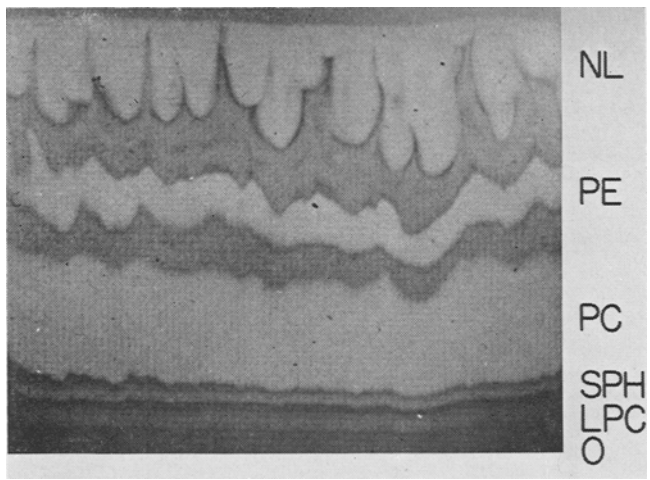

FIG. 3. TLC of a total lipid extract of egg yolk. Plates: 200 × 200 × 0.25 mm silica gel G. Solvent: chloroform-methanol-water (65:25:4, v/v/v). Spray: 0.05% 2,7-dichlorofluorescein in 50% methanol. NL, neutral lipid; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; SPH, sphingomyelin; LPC, PC, lysophosphatidyl choline; O, origin.

are purified further by thin-layer chromatography usually in a solvent system different from that used in the original resolution.

Fig. 3 shows the TLC resolution of a total lipid extract of egg yolk in a phospholipid developing system. Under these conditions all the neutral lipids move with the solvent front. Either the phosphatidyl ethanolamine or the phosphatidyl choline, which are clearly separated from sphingomyelin and lysolecithin, or both can be recovered in high purity. Each phospholipid represents 5 to 10 mg of material, which suffices for the entire analysis. The glyceryl phosphatides are quantitatively recovered from the plates by elution with chloroform-methanol-water (50:40:10) containing a trace of acetic or formic acid.

The purified glyceryl phosphatide is characterized by determining the overall and the positional distribution of fatty acids. For uncommon phosphatides from new sources, additional tests of identity may be required, the discussion of which is beyond the scope of this presentation. Fig. 4 shows the GLC patterns of the total fatty acids of rat heart lecithins along with those found in the 1 and 2 positions. The acids in the 2 position were specifically released by digestion of the purified lecithin mixture with phospholipase A. The methyl esters were prepared by transmethylation (5). Proportional summation of the mole percentages of the acids in the 1 and 2 positions gave a perfect reconstitution of the overall composition indicating that all the molecular species of lecithin were completely hydrolyzed by the enzyme in the allotted incubation time. If desired, the total lecithin preparation could now be subfractionated by silver nitrate TLC and the recovered lecithins subjected to a further analysis of fatty acids.

Preparation of Diglyceride Acetates

These are prepared from free diglycerides which can be quantitatively released from the glyceryl phosphatides by digestion with appropriate phospholipases. Phospholipase C from *Clostridium welchii* has proved to be effective for the hydrolysis of lecithins (4), while the phospholipase C from *Bacillus cereus* has been found satisfactory for the hydrolysis of phosphatidyl ethanolamine (13). The diglyceride parts of phosphatidyl inositols can be released using

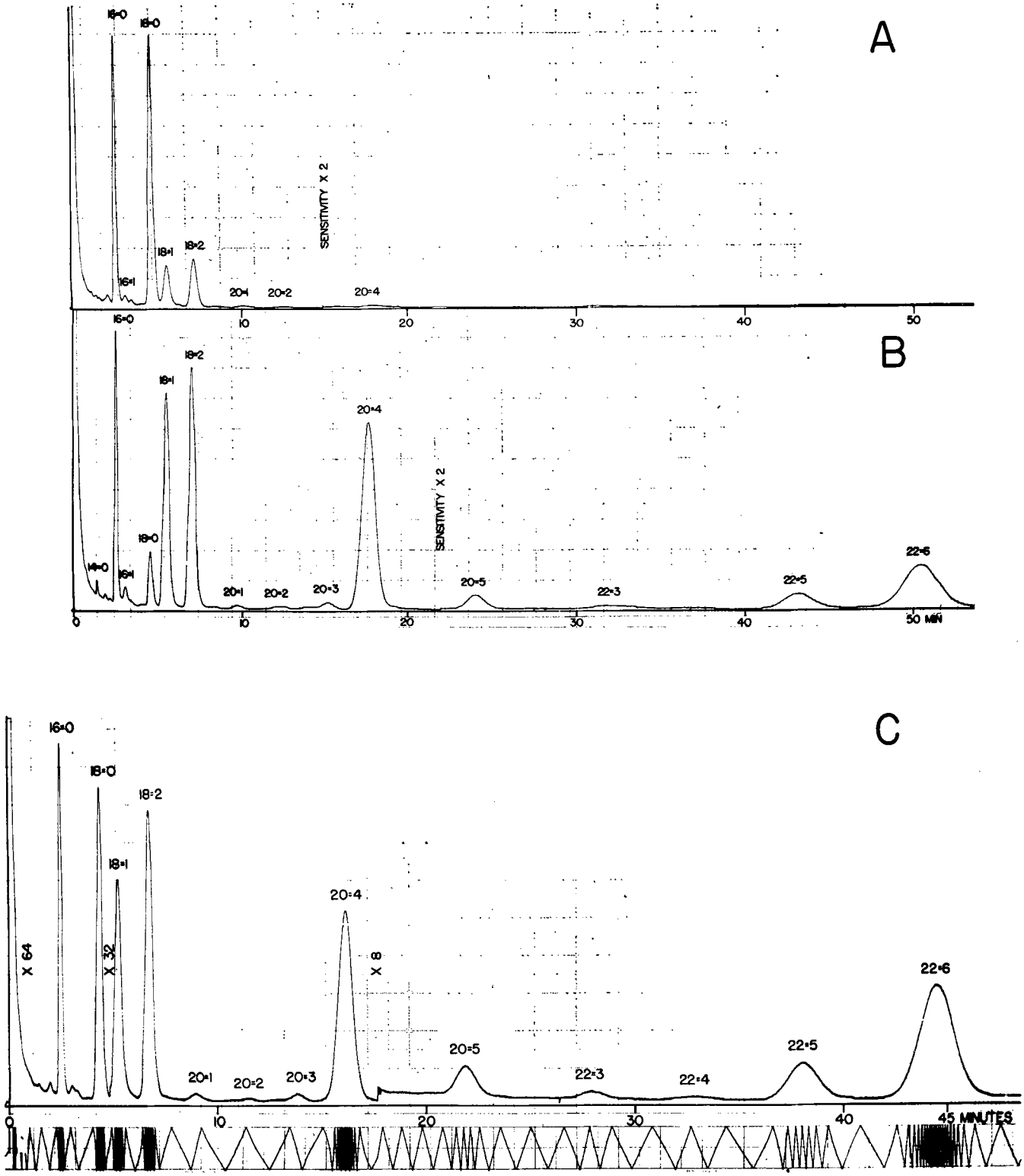

FIG. 4. GLC of the fatty acids of rat heart lecithins, *A*, acids in 1-position; *B*, acids in 2-position; *C*, total acids. Instrument: Varian-Aerograph, Model 204B with on-column injector. Columns: 6 ft × 1/8 in. O.D. stainless steel tubes packed with 10% EGSS-X on Gas-Chrom P, 100–120 mesh (Applied Science Laboratories, Inc., State College, Pa.). Carrier gas: N_2, 50 ml/min. Injector, 210C. Hydrogen flame detector, 240C. Column oven, 180C. Peaks identified as described in the text.

enzyme preparations from brain (14). Although this material has not been extensively purified, representative digestion of over 50% of the material, as judged from the fatty acid composition of the original inositol phosphatide, has been realized. Comparative hydrolyses of phosphatidyl serines have not yet been reported, but phosphatidyl glycerol and cardiolipin have been degraded to diglycerides by digestion with phospholipase C from *Bacillus Cereus* in the presence of Zn ions (15).

These digestions are generally performed under diethyl ether at room temperature, or slightly above, for periods ranging from 30 min to several hours. The recovered diglycerides are purified by TLC and their fatty acid composition determined. If representative of the original fatty acid composition,

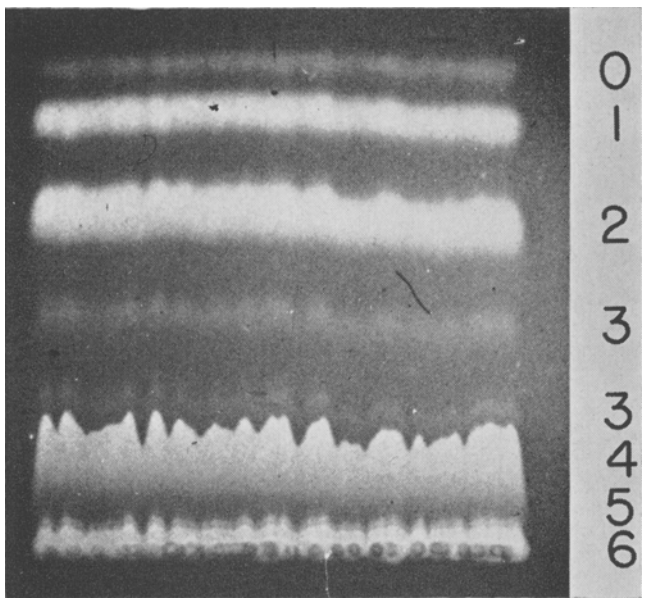

FIG. 5. $AgNO_3$-TLC of the Diglyceride Acetates of Rat Liver Lecithins. Plates: 200 × 200 × 0.5 mm silica gel G impregnated with 20% silver nitrate. Solvent: 0.8% methanol in chloroform. Spray: 0.05% 2,7-dichlorofluorescein in 50% methanol. Bands identified by the degree of unsaturation.

these diglycerides can be subjected to further fractionation by TLC and GLC as described below. For this purpose, however, it is best to convert them into the more stable diglyceride acetates, provided this is not accompanied by isomerization.

The acetates of the diglycerides are prepared by treatment with acetic anhydride and pyridine (10:1, v/v) at room temperature. The use of acetyl chloride is avoided because of potential isomerization of the diglycerides in the presence of HCl. Use of ketene may improve the efficiency of the acetylation step. The diglyceride acetates are purified by TLC and their fatty acid composition again checked by gas chromatography.

Table I gives the estimates of the total fatty acid composition of rat liver lecithins at different stages of analysis and following reconstitution from the final fractionation on the silica gel impregnated with silver nitrate. The close correspondence in the proportions of the acids indicates that complete, or at least representative, recoveries were obtained following all transformations including silver nitrate fractionation. Although these data are derived from work with phosphatidyl cholines, there would appear to be no reason why comparable recoveries of fatty acids should not be obtained with other glyceryl phosphatides and their derivatives.

TABLE I

Recoveries of Total Fatty Acids of Rat Liver Lecithins Under Different Experimental Conditions

Weight %

Fatty acids	Experimental conditions[a]					
	(1)	(2)	(3)	(4)	(5)	(6)
14:0	0.2	0.2	0.4	0.5	0.4	0.5
15:0	0.1	0.2	0.4	0.2	0.3	
16:0	21.4	20.3	20.1	23.9	23.0	24.0
16:1	1.6	1.9	1.4	1.4	1.5	1.5
18:0	19.9	21.5	21.5	18.2	19.0	20.0
18:1	8.8	8.2	7.9	8.9	8.5	9.0
18:2	13.1	12.2	11.3	15.0	13.5	13.8
20:1	0.5	0.5	0.4	0.3	0.4	tr
20:2	0.1	0.2	0.1	0.3	0.3	tr
20:3	0.9	1.0	1.2	1.1	1.2	tr
20:4	19.5	22.7	23.3	20.1	20.5	21.0
20:5 ?	1.7	1.6	1.5	1.5	1.5	tr
22:0 ?	0.2	0.2	0.4	0.3	0.3	tr
22:3	tr	0.2	tr	tr	tr	tr
22:4	tr	tr	tr	tr	tr	tr
22:5	1.1	1.1	0.9	1.0	0.9	1.0
22:6	10.8	8.0	9.4	7.4	7.8	8.6

[a] (1) transmethylation of diglycerides obtained by phospholipase C digestion of total liver lipids.
(2) transmethylation of purified lecithins in presence of silica gel G.
(3) transmethylation of purified lecithins following elution from silica gel G.
(4) transmethylation of diglycerides obtained by digestion of purified lecithins with phospholipase C.
(5) transmethylation of diglyceride acetates obtained from purified diglycerides in (4).
(6) reconstituted composition of fatty acids following $AgNO_3$-TLC of diglyceride acetates.

Preliminary Resolution of Diglyceride Acetates

All variations of the general analytical scheme originally proposed by Renkonen (4) include as an integral part the resolution of the diglycerides on the basis of unsaturation on silver nitrate TLC. Since the GLC separations of diglycerides are largely confined to the differences in molecular weight, an effective preliminary fractionation by argentation TLC is essential. Although free diglycerides have been employed for this purpose (16), due to possible isomerization of the native 1,2- to the 1,3-diglycerides, which possess different migration rates on both TLC and GLC, a conversion to the acetates is preferable. The separations must be carried out rapidly and efficiently.

Fig. 5 shows the $AgNO_3$-TLC resolution of the diglyceride acetates of rat liver lecithins. The TLC system was similar to that of Privett, Blank, Codding and Nickell (17). About 5 mg of total diglyceride acetate was applied to the plate. Following a 40 min development, single bands were obtained for the saturates, monoenes, dienes and tetraenes. The trienes and to a lesser extent the hexaenes were resolved into two bands each. Further resolution of the tetraenes, any pentaenes and hexaenes was achieved by multiple development in the same solvent system or in a system of progressively increasing polarity (5). Between developments, the plates are momentarily removed from the tank and the solvent allowed to evaporate. On subsequent developments the solvent front is allowed to ascend to only about one half the height of the plate. With suitable adjustments in the solvent systems and in the silver nitrate content of the gel (18), it is possible to effect further resolution among most of the diglyceride acetate bands of uniform degree of unsaturation. Thus any monoenes containing trans acids are moved ahead of those containing cis acids, and similar but more complex effects are observed for the dienes and trienes, containing any trans acids. Furthermore, the dienes made up of two monoenoic acids move ahead of the dienes containing one saturated and one diunsaturated fatty acid. The hexaenes containing the saturated and the docosahexaenoic acids are retained slightly longer than those hexaenes which contain the linoleic and archidonic acid pair. Although some of these bands are very small, their identification is important, as this information is necessary to account fully for all types of fatty acid pairings in the original glyceryl phosphatides.

Comparable resolutions are obtained for the diglyceride acetates prepared from lecithins of other tissues, and there appears no reason why this system should not work for the diglyceride acetates derived from all glyceryl phosphatides, which contain similar fatty acids.

GLC of Diglycerides and Diglyceride Acetates

The suitability of short thin-film columns for the GLC separation of the free diglycerides derived from

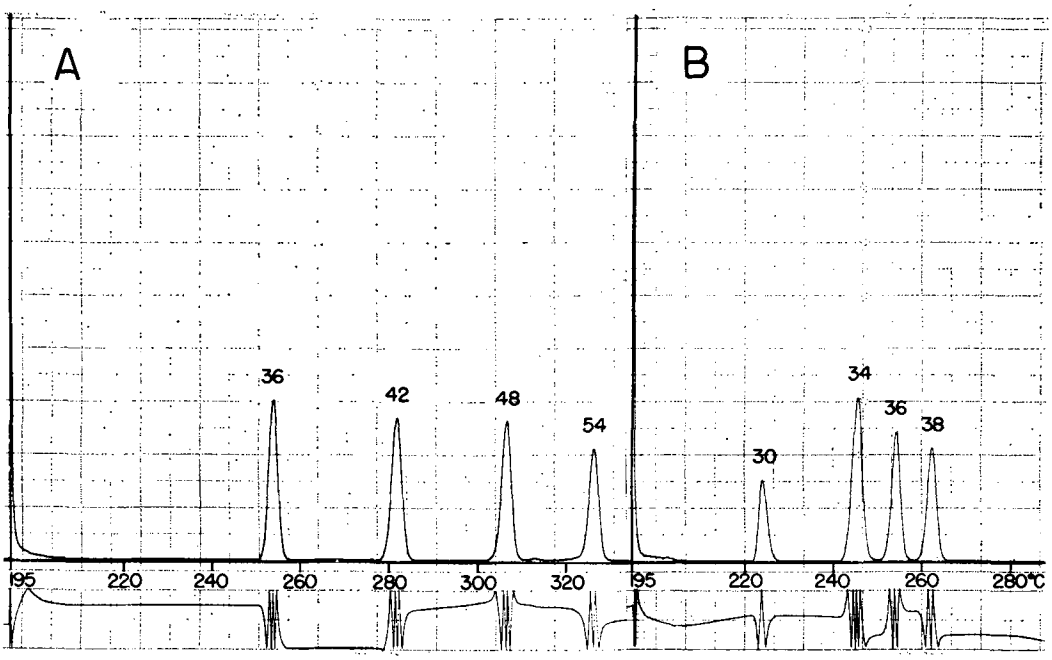

FIG. 6. GLC of simple triglycerides (*A*) and diglyceride acetates (*B*). Instrument: Beckman GC-4 Gas Chromatograph with specially modified on-column injector. Columns: 18 in. × ⅛ in. O.D. stainless steel tubes packed with 3% JXR on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.). Carrier gas: N_2, 150 ml/min. Injector, 300C. Hydrogen flame detector, 350C. Temperature program: as given in the figures. Peaks identified by the total number of acyl carbon atoms.

the phospholipase C hydrolysis of lecithins and from other natural sources, was demonstrated by Kuksis (19). Except for a somewhat lower detector response when compared to triglycerides, no special problems anticipated from dehydration and heat isomerization were noted. Prior to this, Huebner (20) had observed that free diglycerides could not be eluted from a column containing 23% silicone grease, but that acetylation assured their complete elution. Subsequent work in our laboratory has confirmed the superiority of the acetates for the GLC resolution of diglycerides.

Both trifluoroacetylation and silylation are also satisfactory for the preparation of diglyceride derivatives for GLC (21). These compounds, however, are sensitive to hydrolysis in the presence of moisture and cannot be stored. Furthermore, their suitability for $AgNO_3$-TLC and lipase digestion has not been investigated, and it is not known to what extent these derivatives can be incorporated into the general scheme of analysis. The silyl ethers of diglycerides may have special advantages for the GLC resolution of isomeric diglycerides and saturated and unsaturated diglycerides of the same carbon number.

Method of Analysis

The GLC separations of free diglycerides or diglyceride acetates are performed under the improved conditions described for GLC of triglycerides (22). Stainless steel columns, ⅛ in. O.D. × 18 to 24 in. in length, have proved very satisfactory when packed with 1–3% JXR on Gas-Chrom Q, 100–120 mesh (Applied Science Laboratories, Inc., State College, Pa.). These are used after preconditioning for 2–3 hours at 350C, although a gradual further improvement in the column performance may be noted during the next two or three weeks of operation. Most gas chromatographs capable of high temperature operation and equipped with a linear temperature programmer are suitable. Dual column operation is an advantage but not a necessity. Attention is called to the special placement of the injection end of the column into the injector heater (22). This feature has been found to be critical for reliable analysis of both triglycerides and diglycerides. It is thought that this column configuration allows the most effective delivery of the glyceride mixture to the column packing. Gas chromatographs with large dead volumes and/or elbows in this area of the instrument give erratic results.

Separation of Standards

Fig. 6 compares the GLC elution patterns of simple triglycerides (a) and diglyceride acetates (b) recorded under similar experimental conditions. The peak numbers refer to the total carbon atoms in the acyl moieties of these glycerides. When injected together, the diglyceride acetates with 36 acyl carbons overlap completely with trilaurin and there is similar overlap between other triglycerides and diglyceride acetates of corresponding acyl carbon number. For this reason the diglyceride acetates have been assigned a carbon number which also includes the two carbon atoms of the acetic acid residue, rather than the carbon atoms of the two true fatty acids only. The elution patterns are characterized by complete return of the recorder pen to the base line between any two diglyceride acetates differing by two carbon atoms. Under these conditions, both triglycerides and diglyceride acetates are recovered in their weight proportions with a relative error of less than 2%.

The GLC resolution of free diglycerides on such columns is equally satisfactory as seen from Fig. 7a. The peak numbers in this case refer to the fatty acid carbons in the simple saturated diglycerides. In contrast to the chromatograms of the acetates, the runs with the free diglycerides show peaks for by-products of both lower and higher molecular weights than those of the starting materials. The lower molecular weight components have been identified as free fatty acids and monoglycerides, while those of the higher molecular weight correspond to the mixed acid

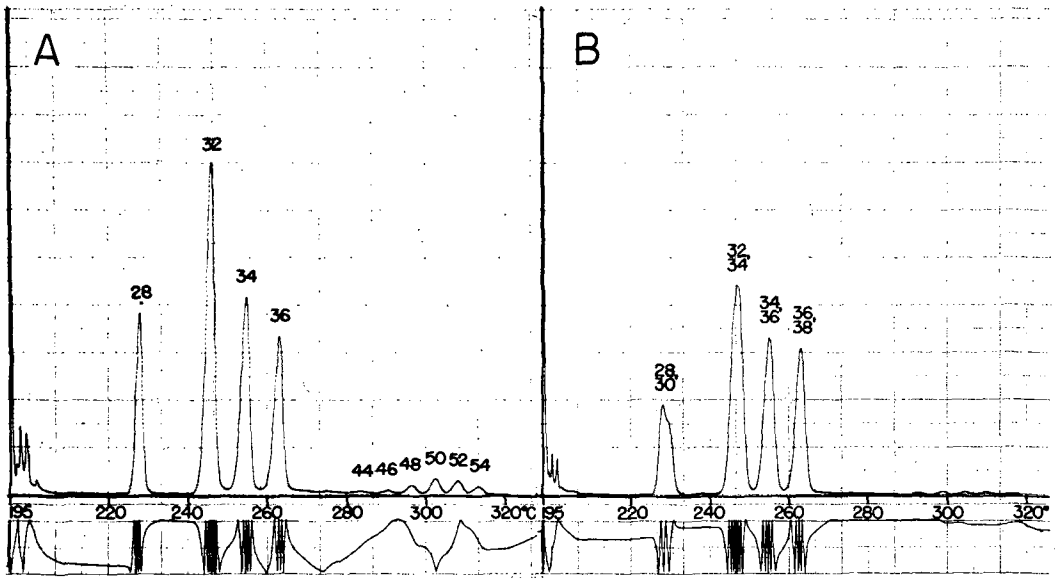

FIG. 7. GLC of free diglycerides and diglyceride acetates. *A*, diglycerides; *B*, equal weight mixture of diglycerides and diglyceride acetates. Experimental conditions and peak identification as in Fig. 6.

triglycerides. Apparently interglyceride acyl migration takes place when free diglycerides are subjected to GLC. On the basis of GLC runs in the presence of internal standard, it has been estimated that the transacylation affects up to 10% of the total material. It is evident that new diglycerides are not formed to any significant extent, as in such a case a peak with a carbon number of 30 would have been detected, representing a diglyceride of myristic and palmitic acids, which was deliberately omitted from the standard mixture. The triglycerides (C_{44}–C_{54}) range from dimyristoyl-monopalmitin all the way to the tristearin, with the dipalmitoyl-monostearin (C_{50}) being the major component. Despite this partial degradation and isomerization, GLC of the free diglycerides, can provide a relatively accurate account of the molecular weight distribution (see below).

Fig. 7b shows the resolution obtained for an approximately equal weight mixture of the standard free diglycerides and their acetates. Except for the dimyristin which migrates slightly faster than its acetate, there is complete overlap among the free diglycerides and the corresponding diglyceride acetates. Under these experimental conditions then, one hydroxyl group is equivalent to two methylene units in retention time. Although a somewhat more complete resolution of the free and acetylated diglycerides can be obtained on longer columns (6 ft), this method cannot be used for a reliable demonstration of incomplete acetylation of diglycerides. On these short columns, there also is no resolution of the 1,2- and the 1,3-diglycerides or their acetates. The isomeric diglycerides can be partially resolved by such columns following the preparation of the silyl ethers or trifluoroacetates (21). In the present analytical system, however, there is no need for the resolution of either the free and acetylated diglycerides or their positional isomers, as both are readily separated by TLC (4) which precedes the GLC step.

Of greater interest is the GLC resolution of saturated and unsaturated diglycerides, as it might provide an alternative to the argentation TLC. Although triglycerides and diglyceride acetates differing by three double bonds can be readily resolved even on nonpolar columns of sufficient lengths (5 ft), not enough work has been done to fully appraise the possibilities for a practical resolution of glycerides on the basis of unsaturation.

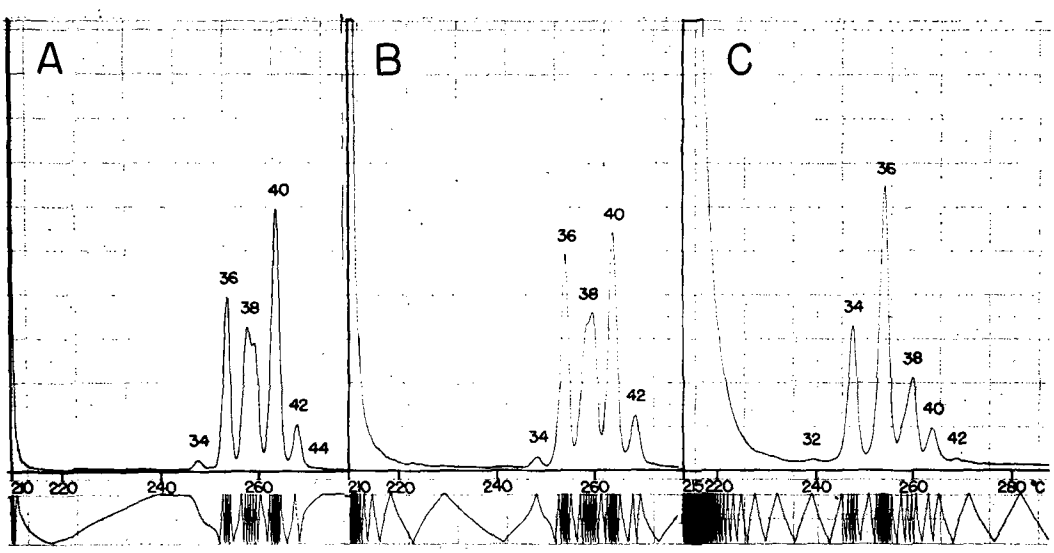

FIG. 8. GLC of diglyceride acetates from rat tissue lecithins. *A*, liver; *B*, plasma; *C*, erythrocytes. Experimental conditions and peak identification as in Fig. 6.

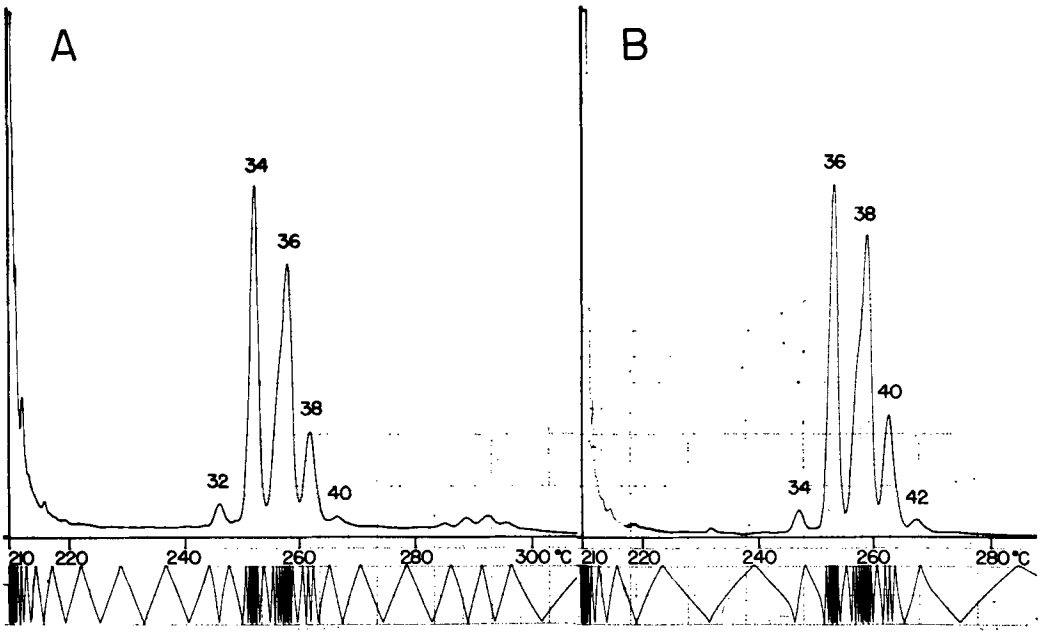

FIG. 9. GLC of free diglycerides (*A*) and diglyceride acetates (*B*) of Lecithins of Rat Intestine. Experimental conditions and peak identification as in Fig. 6.

Separation of Unknowns

Fig. 8 shows the GLC separations recorded for the total diglyceride acetate mixtures obtained from the lecithins of rat liver, plasma and red blood cells. All three elution patterns are composed of diglyceride acetates containing 34 to 42 acyl carbons and represent diglycerides ranging from dipalmitin (C_{34}) to stearoyl docosahexaenoin (C_{42}). The carbon numbers of these peaks have been derived from cochromatography of the unknowns with standard diglyceride acetates. While the diglyceride acetates of the plasma lecithins are very similar to those of the liver lecithins, the diglyceride acetates of the red blood cell lecithins are different. These contain proportionally more of the saturated shorter chain derivatives than either liver or plasma lecithins. All three elution patterns show a shoulder for peak 38. This is due to the presence of palmitoyl arachidonoin in these peaks, which migrates faster than the acetates of the dioctadecanoins of different degrees of unsaturation also present.

Despite the high proportion of arachidonic and the docosahexaenoic acids in the liver lecithins, the derived diglyceride acetates show no signs of degradation during the GLC. All acetates are eluted in their weight proportions as shown by the identity of the GLC patterns recorded before and after hydrogenation. As shown elsewhere (23), the diglyceride acetates are recovered in the proportions anticipated on the basis of the fatty acid composition of the original lecithins.

Fig. 9 compares the GLC elution patterns recorded for the free and acetylated diglycerides of the lecithins of the rat intestine. The elution patterns are essentially identical except for the small amounts of the mono- and triglycerides again formed during the chromatography of the free diglycerides. In view of the similarity in the proportions of the major peaks, there appears to have been little interglyceride acyl migration. As noted in Fig. 8, the diglycerides contributing to peaks 36 and 38 are partially resolved due to the presence of palmitoyl arachidonoin in these peaks.

The GLC analysis of the diglyceride acetates is

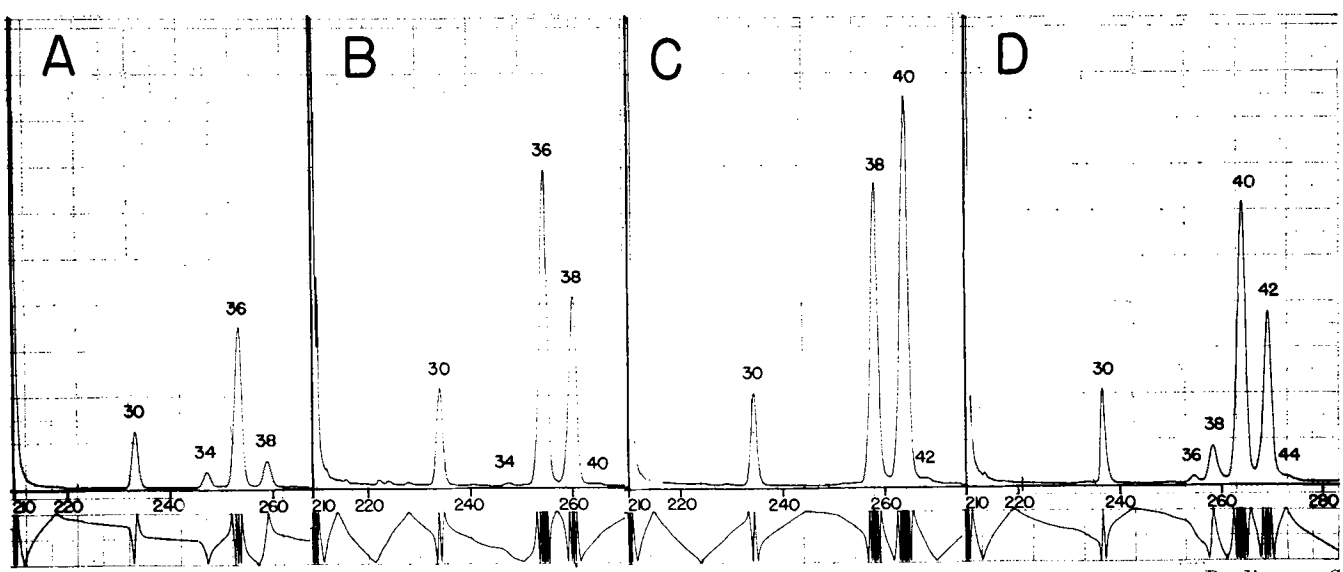

FIG. 10. GLC of diglyceride acetates of uniform degree of saturation from rat liver lecithins. *A*, monoenes; *B*, dienes; *C*, tetraenes; *D*, hexaenes. Experimental conditions and peak identification as in Fig. 6. Peak 30, tridecanoin internal standard.

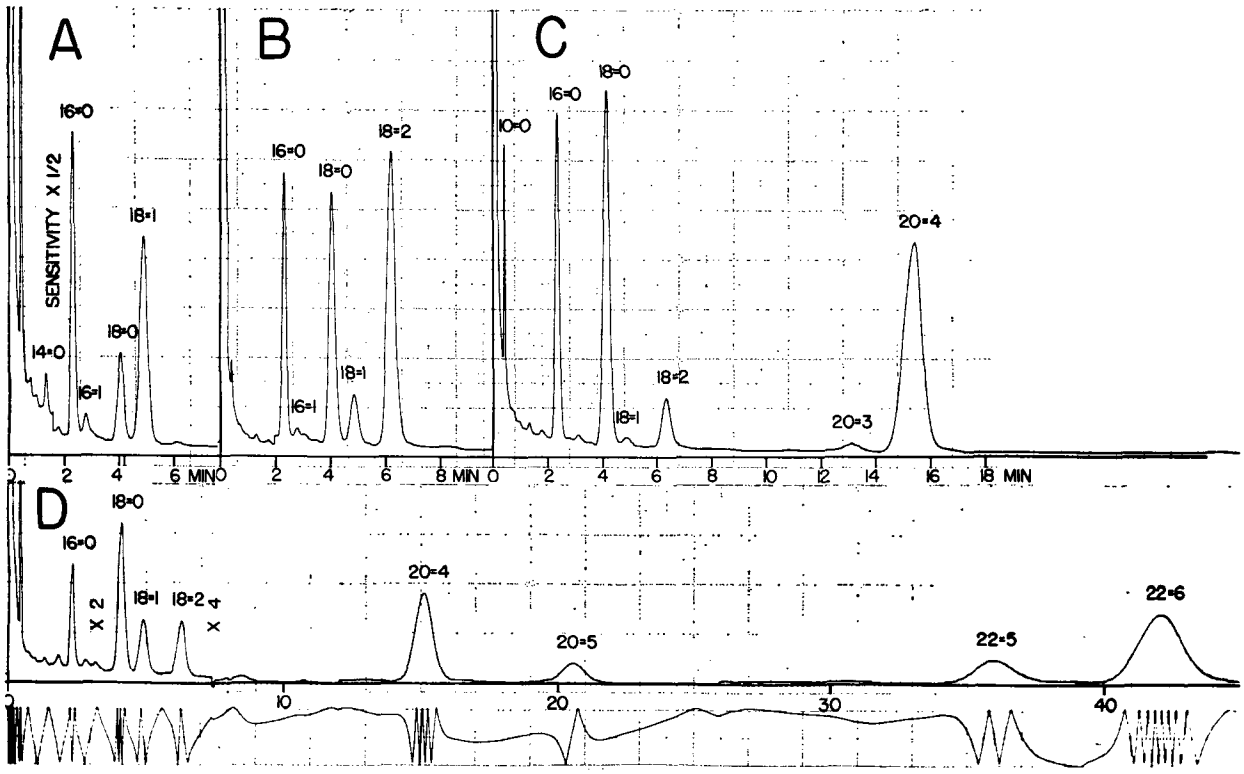

FIG. 11. GLC of fatty acids of diglyceride acetates of uniform degree of saturation from rat heart lecithins. *A*, monoenes; *B*, dienes; *C*, tetraenes; *D*, hexaenes. Experimental conditions and peak identification as in Fig. 4.

most effective following a preliminary resolution of these derivatives on the basis of unsaturation on silica gel impregnated with silver nitrate. Fig. 10 shows the major diglyceride acetates of the rat liver lecithins recovered from argentation TLC. The monoenes (a) are seen to be made up of components with a total acyl carbon number of 36 and 38, which, on the basis of the fatty acid composition reported elsewhere (23), represent largely palmitoyl and stearoyl oleins. The dienes (b) contain mostly palmitoyl and stearoyl linoleins and some dioleins and give two peaks also with total acyl carbon numbers of 36 and 38. The tetraenes (c) contain palmitoyl and stearoyl arachidonoins with total acyl carbon numbers of 38 and 40. The hexaenes (d) show a more complex pattern, but major contributions by the palmitoyl (C_{40}) and stearoyl (C_{42}) docosahexaenoins are apparent. Peak 30 represents tridecanoin, which has been added to the TLC fractions as an internal standard for the estimation of the proportions of the fractions. Other minor bands, such as the saturates, trienes and pentaenes show equally well defined diglyceride acetate peaks, the molecular weight of which can again be established from the relative retention time. Determination of the exact peak composition requires accurate fatty acid analyses, which are best performed on the various TLC fractions following rechromatography.

GLC of Fatty Acid Methyl Esters

Accurate GLC analyses of fatty acids require suitable columns and effective instrumentation. Separations based on the molecular weight and unsaturation of the fatty acids can be achieved with conventional polyester columns and are sufficient for the analyses of the common fatty acid methyl esters. Eventually, however, means may have to be found for the simultaneous resolution of geometric and positional isomers of unsaturated fatty acids as well as acids containing hydroxyl groups and cyclopropane rings.

Identification of Fatty Acids

Detailed discussion of the techniques employed in the identification of fatty acids by GLC is beyond the scope of this presentation. For the present purposes it has proved sufficient to separate and identify the acids on the basis of their retention times on a single polyester column before and after hydrogenation of the sample. The segregation of the diglyceride acetates on the basis of unsaturation on the silver nitrate plates provides further clues regarding the identity of various minor fatty acid peaks. Where doubt remains, it has been most profitable to subject the methyl esters of the fatty acids to a further resolution on silver nitrate treated silica gel on the basis of both unsaturation and chain length (18). By these means it has been possible to overcome to a large extent the lack of pure standards of polyunsaturated fatty acids. The use of the methyl esters of the mixed polyunsaturated fatty acids obtained by transmethylation of ordinary cod liver oil as a source of reference compounds (24) has also been effective.

Fig. 11 shows the GLC elution patterns recorded for the fatty acids of the monoenoic, dienoic, tetraenoic and hexaenoic diglyceride acetates of rat heart lecithins following recovery from argentation TLC. As shown elsewhere (25) the above described means were fully adequate for the resolution and reliable identification of all the characteristic acid components of these fractions.

Quantitation of Fatty Acids

In addition to correct identification, perfect pairing of the fatty acids and accurate reconstitution of

TABLE II

Reconstitution of the Composition of the Monoenoic Lecithin Fraction from Egg Yolk

Experimental values						Calculated values[a]		
Glycerides	Mole %	Fatty acids	Mole %			Lecithins		Mole %
			Total	α	β	α	β	
34	2	16:0	40	80		16:0	16:1	2
		16:1	1.5		3	16:0	18:1	77
36	78	18:0	10	20		18:0	16:1	1
		18:1	48		96	18:0	18:1	19
38	20	20:1	0.5		1	16:0	20:1	1
						18:0	20:1	Trace
Calculations:			34 = (16:0 16:1) = 2% 36 = (16:0 18:1) + (18:0 16:1) = 78% Since (18:0 16:1) = 1%, (16:0 18:1) = 77% 38 = (18:0 18:1) + (16:0 20:1) = 20% Since (16:0 20:1) = 1%, (18:0 18:1) = 19%					

[a] The amounts in which these lecithin species occur in the original lecithin mixture is obtained by multiplying the calculated values by the percentage of the monoene fraction.

the original lecithin composition requires quantitative data of the acid composition. While comparisons of the proportions of standards usually produce adequate results in the hands of experienced operators, there are inherent dangers in assuming that quantitation in GLC is justified for all classes of compounds when only one class, such as the esters of saturated fatty acids, is verified. It has been shown (26) that, for example, only saturated and monounsaturated fatty acids can be adequately quantitated on open tubular columns, the analyses of long chain polyunsaturated fatty acids of marine origin being accompanied by serious losses. Some loss of the polyunsaturated fatty acids would also be anticipated on packed polyester columns, particularly when working at elevated temperatures and employing carrier gasses with significant oxygen contamination. It is therefore necessary to use calibration factors or response curves. The true content of the polyunsaturated fatty acid in the mixture is obtained by GLC after hydrogenation, assuming that the fully saturated acid is completely eluted and its response in the flame ionization detector is the same as that of the unsaturated parent compound. These assumptions have proven to be correct in our laboratory, within the experimental error at least, and have permitted complete matching of the fatty acid pairs recovered from the TLC separations. Other types of GLC apparatus, column packings, detectors and variations in technology require independent verification of the quantitative response of the analytical system.

In practice the pairings of the fatty acids, following the recovery of the diglyceride acetates from the silver nitrate plates, are limited to relatively few possibilities and strict accuracy in the fatty acid analysis may be unnecessary, since the predominant combinations of the major acids are readily apparent. Such partially guessed combinations, however, severely limit the number of accurately identified molecular species, and as shown by comparisons to random calculations (25,23,29) may occasionally be in serious error.

Calculations

The structure of the original glyceryl phosphatide is calculated by proportional summation and normalization of all the analytical data. The accuracy of the final result depends on proper use of internal standards and any correction factors required by the quantitative technique. Table II illustrates the methods used in the reconstitution of the original composition of the monoenoic lecithins of egg yolk. This calculation can be extended equally well to the tetraenes and hexaenes. The dienes and any trienes and pentaenes require more complicated manipulations as it is necessary to correct for the presence of two or more different fatty acids in the 2-position. This may require several trials and errors before a correct estimate is derived. Occasionally, however, fatty acid combinations are encountered which do not permit an exact solution, as the analytical information is insufficient to provide one equation for each unknown. In such cases further resolution must be sought by some other means (see Ref. 27, for a discussion). Fortunately, even then it is usually possible to obtain a reliable account of the major molecular species insuring the success of the overall analysis.

To guard against errors in positional assignment of fatty acids, and mistakes in summations, the calculations include the following independent and essential algebraic cross-checks. First, the composition and positional distribution of the fatty acids in the original lecithin mixture is matched against the positional distribution and overall composition of the fatty acids in the diglyceride acetates. In practice this means that the estimates of specific fatty acid distribution derived from phospholipase A hydrolysis are matched against the specific acid distributions obtained from pancreatic lipase digestions. Second, the mole proportions of the diglyceride acetates in the original mixture and in the fractions derived from the silver nitrate TLC are matched against the mole proportions of the fatty acids in these fractions and where necessary appropriate corrections made. Third, the accuracy of the estimates is tested by reconstitution of the diglyceride acetate and fatty acid composition from the silver nitrate fractionation. Finally, a further check on the proportional distribution and the possible pairings of the fatty acids and diglyceride acetates is provided by the use of the random calculation (28) at appropriate stages of data evaluation.

Table III shows the nature of the information that can be obtained from such an analysis. These data were obtained for duplicate determinations of the lecithin composition of the red blood cells of man (29). The relative error is 2–5% for the major components (more than 10% of the total) but may exceed 10% for minor components (less than 10% of the total). In view of the extensive fractionation and elaborate reconstitution this agreement must be considered excellent.

Incorporation of measurements of radioactivity in this analytical system would be an obvious advantage. This could be readily accomplished by equipping the gas chromatographs with radioactivity monitoring

TABLE III

Major Lecithins of the Erythrocytes[a]
(Moles %)

Chemical classes	Fatty acids		Lecithins		Chemical classes	Fatty acids		Lecithins	
	α	β	(A)	(B)		α	β	(A)	(B)
	14:0	16:0	6.8)	6.6)		14:0	20:5	)	2.0)
	14:0	18:0	4.6)	6.6)		14:0	22:5	)	2.0)
Saturates	16:0	16:0	69.5)5	69.7)5		16:0	20:5	)	18.8)
	18:0	16:0	19.1	17.1)		16:0	22:5	)	12.5)
						18:0	20:5	)	4.1)
	14:0	18:1	0.5)	1.0)		18:0	22:5	)	4.1)
	16:0	16:1	2.1)	3.0)		16:1	20:4	)	3.5)
Monoenes	16:0	20:1	1.0)35	Tr)37		16:1	22:4	)	0.6)
	16:0	18:1	80.6)	78.7)		18:1	20:4	)	30.3)
	18:0	16:1	2.4)	4.5)	Pentaenes	18:1	22:4	)	4.1)3
	18:0	18:1	13.3)	12.9)		18:2	20:3	)	4.1)
						18:2	22:3	)	10.0)
	14:0	18:2	0.9)	)		20:1	20:4	)	0.6)
	14:0	20:2	2.2)	)		20:1	22:4	)	0.6
	16:0	18:2	61.7)	60.5)		20:2	20:3	)	1.0)
	16:0	20:2	2.7)	0.1)		20:2	22:3	)	2.0)
	18:0	18:2	22.4)	26.9)				)7	
Dienes	18:0	20:2	0.5)35	0.7)32		12:0	22:6	)	1.4)
	16:1	16:1	1.0)	Trace		14:0	22:6	)	2.1)
	18:1	16:1	3.1)	2.1)		16:0	22:6	)	59.5)
	18:1	18:1	4.9)	7.2)		18:0	22:6	)	14.2)
	18:1	20:1	Tr	Tr)		16:1	20:5	)	0.7)
						16:1	22:5	)	1.1)
	14:0	20:3	0.8)	1.7)		18:1	20:5	)	1.4)
	16:0	20:3	52.1)	52.2)		18:1	22:5	)	2.8)
	18:0	20:3	15.1)	11.7)		18:2	20:4	)	9.9)
Trienes	16:1	18:2	4.4)5	5.2)6	Hexaenes	18:2	22:4	)	5.7)4
	18:1	18:2	25.9)	25.2)		20:1	22:5	)	Tr)
	18:1	20:2	0.7)	)		20:1	20:5	)	Tr)
	20:1	18:2	0.7)	0.4)		20:2	20:4	)	1.0)
						20:2	22:4	)	Tr)
	14:0	20:4	1.0)	0.6)					
	16:0	20:4	66.0)	65.0)					
	18:0	20:4	25.5)	28.7)					
	20:0	20:4	0.5)	Tr)					
Tetraenes	16:1	20:3	1.0)13	Tr)13					
	18:1	20:3	2.0)	2.2)					
	18:1	22:3	Tr)	0.6)					
	18:2	18:2	4.0)	3.4)					

[a] Estimated best fit only, not absolute identities or proportions. A and B are parallel analyses of the same sample. Pentaenes and hexaenes in A were not completely resolved, hence a combined estimate only has been entered.

systems. As a result, the present rather static account would assume a dynamic significance, since along with the mass proportions it would be possible to calculate also the specific activities of each fatty acid in any one molecular species of the glyceryl phosphatides.

Possible Significance of Analysis

Studies with hepatic lecithins have demonstrated a structural heterogeneity on the basis of silver nitrate fractionation (3) and a metabolic heterogeneity by marked differences in the relative rates of incorporation of phosphate (1,3,30), choline (3), and the methyl group of methionine (2,3,30) into the linoleoyl and arachidonoyl lecithins. As a result it has been suggested that the polyunsaturated fatty acids exert considerable selectivity in the saturated fatty acids with which they pair in the lecithin molecule (1,2,3,30), and that these specific pairings bear a direct relation to the metabolic behavior of the lecithin (31). Furthermore, different rates and possibly different pathways of synthesis have been suggested (30) for the different species of lecithins. There is evidence (32) that a comparable situation exists in regard to the metabolic activity of other glyceryl phosphatides.

In view of the demonstrated presence of enzymes, which are capable of distinguishing between the 1 and 2 positions of the lecithin molecule and between saturated and unsaturated fatty acids, it has been postulated (33,34) that the variations in the dynamic state of the lecithin molecules are related to their chemical structure rather than differences in metabolic pools. It seems therefore appropriate in future studies, to approach the metabolism of glyceryl phosphatides as individual molecular species rather than specific phospholipid classes.

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