Four-directional-development thin-layer chromatography of lipids using trimethyl borate

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ABSTRACT Solvent mixtures containing trimethyl borate virtually eliminated the pronounced interconversion of 1,2- and 1,3-dipalmitins during their resolution by thin-layer chromatography on Silica Gel G. With trimethyl borate, an average of 1–2% of 1,2-dipalmitin was converted to 1,3-dipalmitin. A four-directional-development TLC procedure incorporating trimethyl borate resolves cholesteryl glucoside, ceramides, monogalactosyl diglyceride, 1- and 2-monopalmitin, palmitic acid, cholesterol, 1,2- and 1,3-dipalmitin, tripalmitin, methyl palmitate, cholesteryl palmitate, β-carotene and some of its degradation products, squalene, and tetracosane. Digalactosyl diglyceride, phosphatidic acid, phosphatidylglucose, cerebrosides, and other phospholipids remain near the origin. A mixture containing triolein, 1,2- and 1,3-diolein, 1- and 2-monoolein, oleic acid, and cholesterol was resolved in one dimension. A similar series of palmitic-containing neutral lipids was also resolvable in one dimension. These procedures were applied to the TLC of human sera lipids.

SUPPLEMENTARY KEY WORDS 1,2-dipalmitin · 1,3-dipalmitin · 2-monopalmitin · 2-monoolein · borate · isomerization

THIN-LAYER chromatographic procedures are available for the resolution of neutral lipids in one dimension by one- and two-step development (1–5). Using these procedures, we have experienced difficulty in separating glycerides, cholesterol, fatty acids and their esters, hydrocarbons, and other lipids on a single 20 × 20 cm TLC plate. Furthermore, we have observed significant interconversion of 1,2-dipalmitin and 1,3-dipalmitin during TLC. This study describes a four-directional-development TLC procedure which can resolve complex mixtures of lipids containing neutral lipids, glycolipids, and phospholipids and also eliminate the interconversion of diglycerides.

METHODS

Lipids were chromatographed on commercially prepared Silica Gel G-coated aluminum sheets (6820000-8 or 6820200-1, Brinkmann Instruments, Westbury, N.Y.) or on coated glass plates (Uniplates, Analtech, Inc., Wilmington, Del.). Glass plates were precleaned by chromatography with chloroform and then benzene–ethyl acetate 100:20.

For four-directional-development TLC we used four solvent systems: in the first direction, chloroform–ethanol–trimethyl borate (CEB) 100:1:6; in the second, benzene–ethyl acetate–trimethyl borate (BEAB) 100:20:7.2; in the third, heptane–benzene (HB) 60:40; and in the fourth, heptane (H). In the final procedure after each TLC direction, plates were air dried in a hood at room temperature (21–24°C) for 45 min, rotated 90°, and chromatographed in the next direction. Plates were chromatographed in each of the first three directions for about 18 cm, and in the fourth direction, using heptane, for about 10 cm.

To resolve phospholipids on the same plate, the fourth direction and solvent were eliminated; plates were chromatographed in the first three directions, as above, and rechromatographed in the second direction using chloroform–methanol–water (CMW) 70:20:2.5. Phospholipids are practically immobile in the first three solvent systems (CEB, BEAB, HB), and are localized at the origin. Before rechromatography in the second direction with CMW, the phospholipid lane was isolated by scraping away silica gel (see Fig. 5).

Lipids were visualized by spraying the plates with rhodamine 6G or by charring after sulphuric acid–dichromate spray (6). Trimethyl borate contained a contaminant visualized by charring which was immobile

Abbreviations: TLC, thin-layer chromatography.

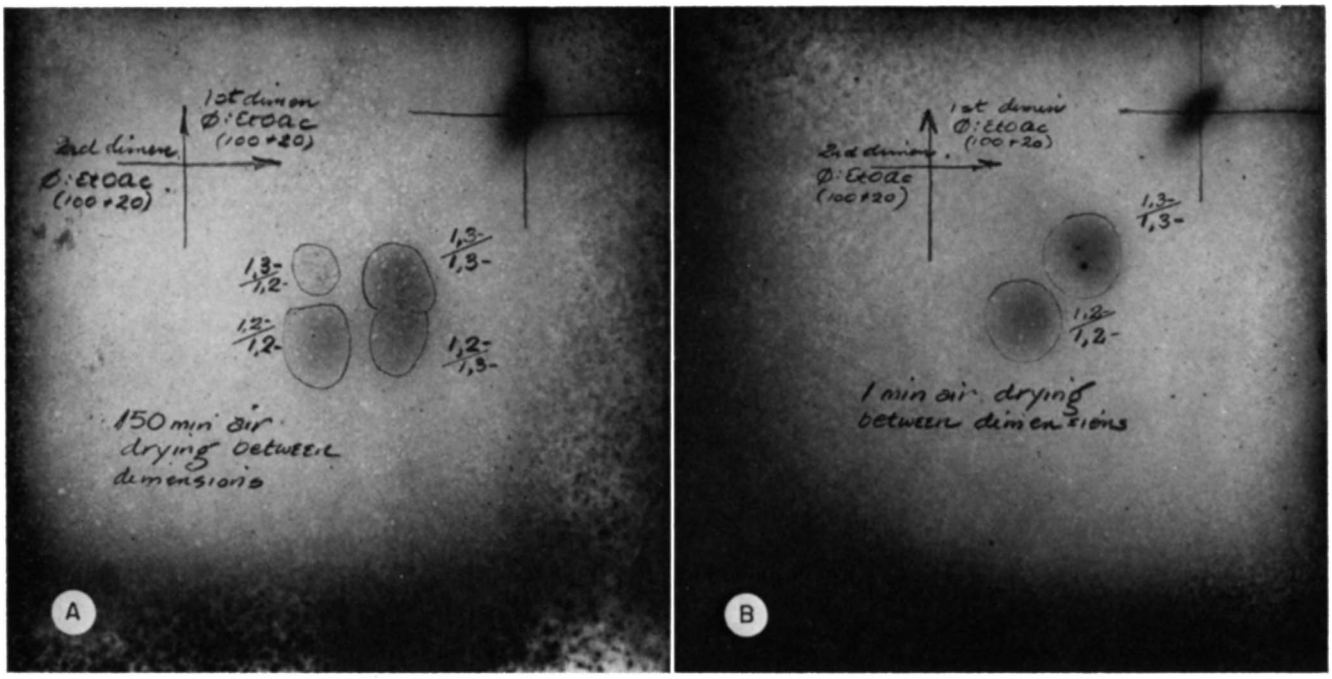


FIG. 1. Thin-layer chromatograms of 200 μg each of 1,2- and 1,3-dipalmitin on Silica Gel G-coated aluminum sheets. First and second direction developing solvents, BEA. Spot *1,3/1,3* is 1,3-dipalmitin. Spot *1,2/1,3* is also 1,3-dipalmitin, but arising from 1,2-dipalmitin which had resolved in the first direction. Spot *1,2/1,2* is 1,2-dipalmitin. Spot *1,3/1,2* is also 1,2-dipalmitin, but arising from 1,3-dipalmitin which had resolved in the first direction. *A*, interdimension drying time 150 min; *B*, interdimension drying time 1 min.

in the four solvent mixtures, CEB, BEAB, HB, and H. However, this contaminant migrated in CMW with an R_F of about 0.4. Therefore, before using this solvent we removed edges of the plate that had been in contact with solvent containing trimethyl borate, and which would be immersed in CMW.

Dipalmitin spots, either 1,2- or 1,3-, seen with rhodamine, were scraped from the plates and extracted three times at 37–40°C with about 2 ml of chloroform–diethyl ether 1:1. The extracts were pooled, filtered, and then dried at 60°C. Recovery of dipalmitins was 13% less when diethyl ether (4) was used as an extraction solvent.

The dipalmitin residues were quantitated by assay for glyceride (7). In this glyceride assay, equimolar concentrations of 1-monopalmitin, 1,2- or 1,3-dipalmitin, and tripalmitin standards gave the same absorbances over a range of 2–210 nmoles, and the coefficient of correlation between these four standard curves was 0.986 (8). Recovery of dipalmitin standards after two-direction chromatography in CEB and then BEAB was 93 ± 2% (SEM) over the range of 17–173 nmoles. Over the same range, the presence or absence of trimethyl borate in both solvent mixtures made no difference in the recovery of dipalmitin standards (coefficient of correlation was 0.999).

Lipids were extracted from human sera by mixing samples with 20 vol of chloroform–methanol (CM) 2:1; the lipids were purified by the method of Folch, Lees, and Sloane Stanley (9).

Trimethyl borate, triethyl borate, triethanolamine borate, dichlorophenylborane, triphenylborine, phenylboric acid, tetraphenylboron sodium, and triisopropanolamine borate were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisc. Tri-*n*-propyl borate, tri-*iso*-propyl borate, and trimethylamine borane were obtained from Peninsular ChemResearch, Inc., Gainesville, Fla. On the basis of solubility, apparent decomposition, volatility, availability, and effectiveness in preliminary experiments, trimethyl and triethyl borate were selected for further examination.

All standards were obtained from Supelco, Inc., Bellefonte, Pa., Applied Science Laboratories Inc., State College, Pa., or Eastman Kodak Co., Rochester, N.Y. Samples of cholesteryl glucoside and phosphatidylglucose were kindly supplied by P. F. Smith, University of South Dakota, Vermillion. Samples of highly purified β-carotene were kindly supplied by P. Garry, Ohio State University, Columbus.

RESULTS

Resolution and Interconversion of 1,2- and 1,3-Dipalmitin on Silica Gel G-coated Aluminum Sheets

A mixture containing 200 μg each of 1,2- and 1,3-dipal-

mitin was chromatographed on Silica Gel G-coated aluminum sheets in two dimensions using benzene–ethyl acetate (BEA) 100:20. Plates were air dried for 150 min between the two chromatographic runs. After chromatography and visualization, the appearance of two additional spots indicated that conversion took place during the 150-min drying period or during migration in the second dimension (Fig. 1). These unexpected spots were identified as 1,3-dipalmitin arising from 1,2-dipalmitin and a lesser amount of 1,2-dipalmitin arising from 1,3-dipalmitin. By reducing the drying time to 1 min, the interconversion of dipalmitins was significantly reduced, as determined after rhodamine visualization, extraction, and glyceride assay. The conversion of 1,2- to 1,3-dipalmitin was 6.5% after 1 min of drying, and 26.2% after 150 min; conversion of 1,3- to 1,2-dipalmitin was 5.0% and 14.2%, respectively.

Reduction of Dipalmitin Interconversion with BEA Solvent Containing Boron on Silica Gel G-coated Aluminum Sheets and Glass

A mixture containing 200 μg of both 1,2- and 1,3-dipalmitin was chromatographed on Silica Gel G-coated aluminum sheets in two dimensions with BEA solvent. Various concentrations of either trimethyl or triethyl borate were added to the solvent. The lipids were visualized, eluted, and quantitated by the glyceride assay. The percentage conversion of 1,2- to 1,3-dipalmitin was calculated by first adding the amount of glyceride found in the two spots derived from the slower moving 1,2-dipalmitin in the first direction. This sum was considered to be reactant 1,2-dipalmitin. After chromatography in the second direction, the faster 1,3-dipalmitin appearing in the same lane as the reactant 1,2-dipalmitin was assayed and considered to be product. Similarly, the percentage conversion of 1,3- to 1,2-dipalmitin was calculated. The results (Fig. 2) indicated that both trimethyl and triethyl borate substantially reduced interconversions of dipalmitins. Triethyl borate (bp 120°C) appeared more effective at lower concentrations than trimethyl borate (bp 68–69°C); however, it was not as readily removed by evaporation at room temperature. Subsequent experiments showed that the removal of triacyl borates after resolution of the dipalmitins was necessary for subsequent resolution of less polar lipids. Because of its volatility and effectiveness, trimethyl borate was chosen and used to reduce dipalmitin interconversion in all further experiments.

Glass TLC plates were used without drying, spotted with 250 μg of 1,2-dipalmitin, and chromatographed in two dimensions with BEA containing various amounts of trimethyl borate. Lipids were visualized, eluted, and quantitated as described above. A slightly higher concentration of borate was required for maximal

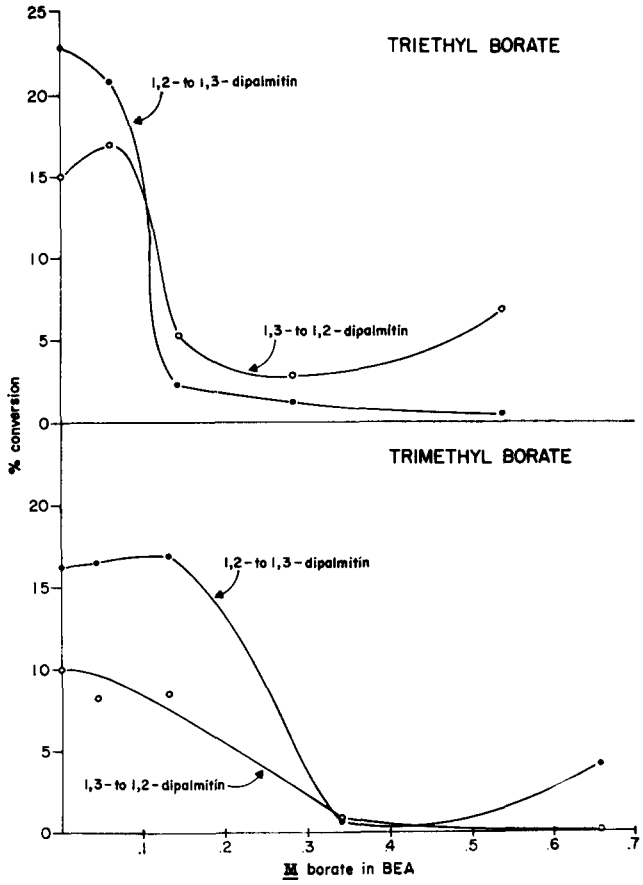


FIG. 2. The effect of borate on the conversion of 1,2- to 1,3-dipalmitin and 1,3- to 1,2-dipalmitin on Silica Gel G-coated aluminum sheets. The developing solvent was BEA which contained various concentrations of either trimethyl or triethyl borate. The aluminum sheets were chromatographed in two directions using the same developing solvent.

reduction of dipalmitin conversion on glass plates (Fig. 3) than that necessary using coated aluminum sheets. On glass plates, the lowest mean conversion of 1,2- to 1,3-dipalmitin was 1.0% and was obtained using 5.7% (0.501 M) trimethyl borate; this mixture is designated BEAB. All further experiments were conducted on the precoated glass plates.

The ability of BEAB to resolve in one dimension a mixture of either oleic- or palmitic-containing neutral lipids is seen in Fig. 4.

Comparison of 1,2-Dipalmitin Interconversion with Either BEAB or Petroleum Ether–Diethyl Ether 60:40 as Solvents

We performed a comparative study with 1,2-dipalmitin using in two dimensions either BEAB or petroleum ether (bp 30–60°C)–diethyl ether 60:40. The latter solvent system is very similar to one used by Privett and Blank (10). These authors used Skellysolve F–diethyl ether 60:40 for the TLC resolution of saturated diglyceride

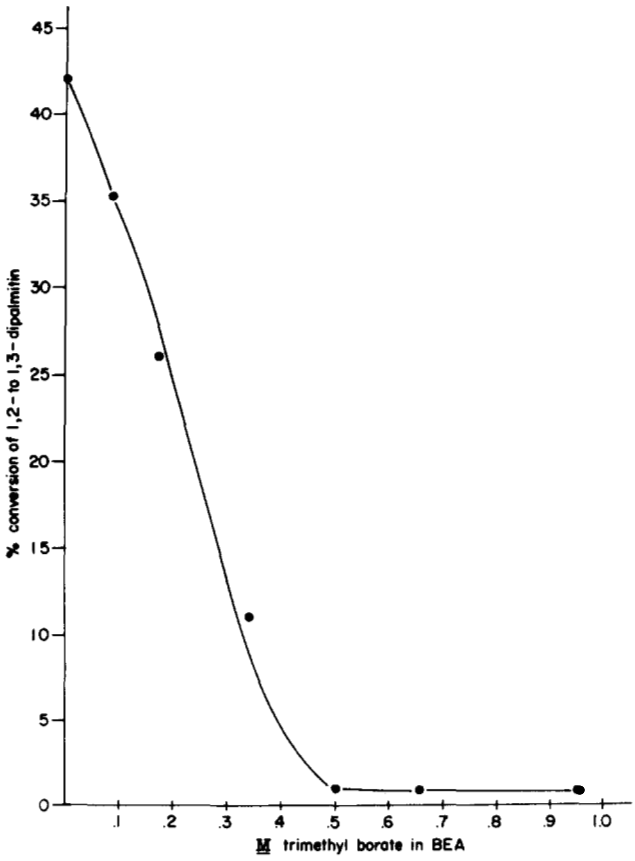


FIG. 3. The effect of trimethyl borate on the conversion of 1,2- to 1,3-dipalmitin on Silica Gel G-coated glass. The developing solvent was BEA which contained various amounts of trimethyl borate. The glass plates were chromatographed in two directions using the same developing solvent.

standards. After chromatography and extraction with chloroform–diethyl ether 1:1, we found that there was only 5.3 ± 0.4% interconversion of 1,2- to 1,3-dipalmitin using BEAB, and 34.8 ± 3.2% using petroleum ether–diethyl ether 60:40.

Four-directional-development TLC

To adequately resolve a complex mixture of lipid standards by TLC, we used a four-directional system of chromatography. Preliminary experiments with another solvent mixture, chloroform–ethanol (CE) 100:1, showed that the addition of trimethyl borate reduced dipalmitin interconversion. The final solvent mixture was composed of chloroform–ethanol–trimethyl borate (CEB) 100:1:6. With CE the conversion of 1,2- to 1,3-dipalmitin averaged 15.6% and with CEB only 0.5%. CEB and BEAB were finally chosen as the first and second direction developing solvents, respectively. Using this solvent sequence, a 45-min "interdimension" drying time, and 400 μg of 1,2-dipalmitin, we found that the average conversion of 1,2- to 1,3-dipalmitin was 1.5%. In the absence of borate in two directions, interconversion could be reduced by lowering the "interdimension" drying time to 2 min. However, due to residual solvent, this brief drying time significantly reduces the resolution of lipids in the second direction. A 45-min drying time between the first and second direction was adopted for all succeeding work. Including initial tank equilibration for 1 hr, the final procedure took about 6 hr.

After four-directional-development TLC, 13 lipid standards could be resolved: cholesteryl glucoside, monogalactosyl diglyceride, 1- and 2-monopalmitin, palmitic acid, cholesterol, 1,2- and 1,3-dipalmitin, tripalmitin, methyl palmitate, cholesteryl palmitate, squalene, and tetracosane (Fig. 5, *A*, *B*); digalactosyl diglyceride, lecithin, and lysolecithin remained at the origin. To resolve these immobile lipids the fourth direction was omitted and the plate was rechromatographed in the second direction with CMW after isolation of the lane (Fig. 5, *C*).

In other experiments, standards of cerebrosides, phosphatidylglucose, phosphatidylglycerol, cardiolipin, sphingomyelin, and other phospholipids migrated only in CMW. Phosphatidic acid was the only phospholipid used which moves in CEB with an R_F of about 0.07 and near the front in CMW.

In solvents used in the first two directions (CEB or BEAB), ceramide samples migrated with R_F values less than that of 1-monopalmitin and were resolvable into six compounds distinguishable from cholesteryl glucoside, monogalactosyl diglyceride, and 1-monopalmitin.

β-Carotene apparently decomposed in this system into at least five visibly yellow components. The most intensely colored and fastest moving substance, presumably β-carotene, moved with the heptane–benzene (60:40) front, and had an R_F of about 0.1 in pure heptane. This substance was intermediate in mobility and was clearly separated from squalene and cholesteryl palmitate. When this component was isolated and rechromatographed, we found an identical picture of five yellow components, of the same relative intensity and R_F as observed on initial TLC.

Resolution of Human Serum Lipids

Lipid from three different serum samples was examined by four-directional-development TLC. One serum was isolated from a normal male subject (*A*). A second sample was recovered from a male patient (*B*) with type IV hyperlipidemia (11). The third serum was obtained from a patient (*C*) diagnosed as having type V hyperlipidemia (11), and who was the father of patient *B*. 1 mg of lipid from each serum sample was chromatographed (Fig. 6). Cholesterol is easily detected as a spherical spot slightly above and to the right of center on all three chromatograms in Fig. 6, and in all other sera examined. In this figure the cholesterol spots

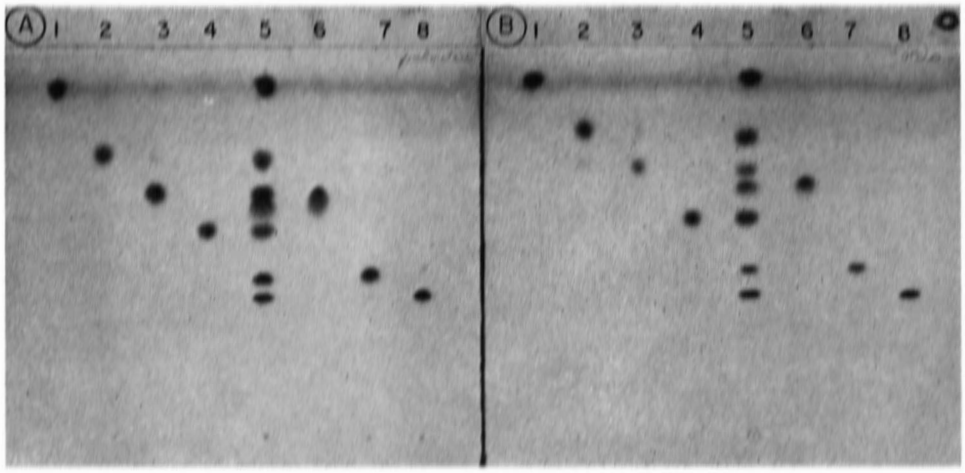
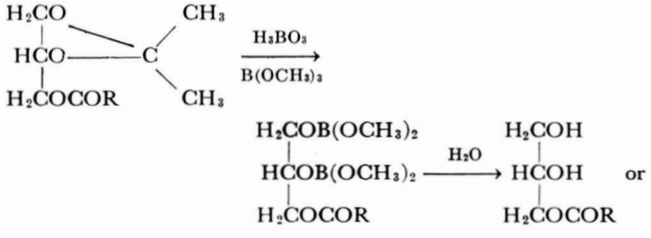


FIG. 4. Thin-layer chromatograms of approximately 35 μg of palmitic or oleic neutral lipids. Adsorbent, Silica Gel G on glass; solvent, BEAB. *A*: lane *1*, tripalmitin; lane *2*, 1,3-dipalmitin; lane *3*, 1,2-dipalmitin; lane *4*, cholesterol; lane *5*, mixture; lane *6*, palmitic acid; lane *7*, 2-monopalmitin; lane *8*, 1-monopalmitin. *B*: lane *1*, triolein; lane *2*, 1,3-diolein; lane *3*, 1,2-diolein; lane *4*, cholesterol; lane *5*, mixture; lane *6*, oleic acid; lane *7*, 2-monoolein; lane *8*, 1-monoolein.

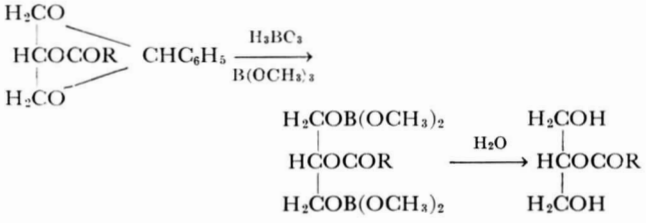
are of comparable intensity. The intensity of the phospholipid spot from serum *A* is obviously greater than that found in sera *B* or *C*. In chromatograms of sera *B* and *C* are found larger, arrow-shaped spots to the right of the cholesterol areas. These distinctively shaped spots are characteristic, in our system, of free fatty acids. Both hyperlipidemic samples contain at least two compounds which are apparently absent from the normal serum. One is seen immediately above the arrow-shaped free fatty acid area, and the other is immediately below and to the left of the cholesterol area. The identity of these lipids is presently unknown, and we have not found components with similar R_F's in other normal sera.

DISCUSSION

Mattson and Volpenhein (12) used trimethyl and triethyl borate for the synthesis of monoglycerides. They found trimethyl borate to be superior. In their procedure, these borates, in the presence of boric acid, replaced the 1,2-isopropylidene or 1,3-benzylidene groups blocking glyceride hydroxyls. The resultant dimethyl borate derivatives were readily hydrolyzed on exposure to water at room temperature to form the 1- and 2-monoglycerides without isomerization. They reported the following reactions:



Wood and Snyder (13) have shown that the 2-isomers of glycerol ethers do not react with arsenite or borate, but the 1-isomers presumably form metal complexes with these ions. The resultant differences in polarity between the complexed 1-isomers and uncomplexed 2-isomers permit their chromatographic resolution. In our work, however, as suggested by the data of Mattson and Volpenhein (12), all free hydroxyls of monoglycerides and diglycerides may be susceptible to derivitization by trimethyl borate. These theoretical borate derivatives (1,2-[dimethyl borate]-3-acyl glyceride; 1,3-[dimethyl borate]-2-acyl glyceride; 1-dimethyl borate-2,3-diacyl glyceride; and 2-dimethyl borate-1,3-diacyl glyceride) are formed during chromatography, and are resolved in our solvent system. Furthermore, they can be quantitated by the glyceride assay and do not undergo acyl migration during TLC.



The four-directional-development TLC procedure has several advantages over other one- and two-dimensional TLC techniques. Classes of neutral lipids, glycolipids, and phospholipids are well separated from each other over a wide area. During chromatography, tetracosane may migrate about 60 cm, cholesteryl and methyl fatty acid esters about 45 cm, triglycerides about 30 cm, etc. The resultant "spread" of complex lipid mixtures improves resolution and increases the possibility of detecting more components.

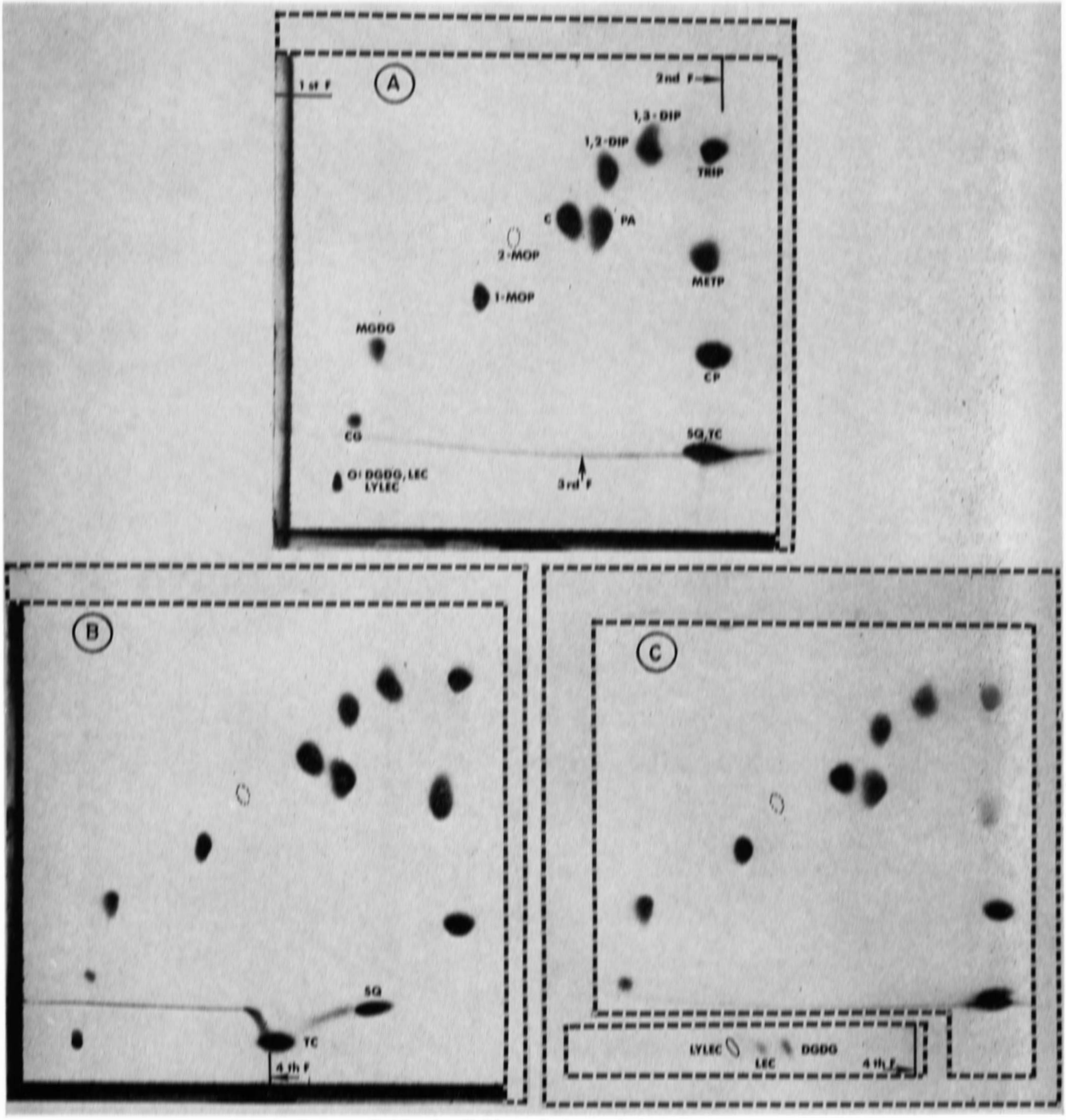


FIG. 5. Thin-layer chromatograms of neutral lipids, glycolipids, and phospholipids resolved by four-directional-development TLC. All Silica Gel G-coated glass plates were spotted with approximately 50 μg of each of 15 standards. Areas devoid of Silica Gel G are outlined. Abbreviations: *LYLEC*, lysolecithin; *LEC*, lecithin; *DGDG*, digalactosyl diglyceride; *CG*, cholesteryl glucoside; *MGDG*, monogalactosyl diglyceride; *1-MOP*, 1-monopalmitin; *2-MOP*, 2-monopalmitin; *C*, cholesterol; *PA*, palmitic acid; *1,2-DIP*, 1,2-dipalmitin; *1,3-DIP*, 1,3-dipalmitin; *TRIP*, tripalmitin; *METP*, methyl palmitate; *CP*, cholesteryl palmitate; *SQ*, squalene; *TC*, tetracosane; *O*, origin; *F*, front. *A*, after three directions. First direction developing solvent, CEB; second direction, BEAB; third direction, HB. *B*, after "standard" four directions. First three directions, unlabeled spots, and fronts as in *A*; fourth direction developing solvent, H. *C*, first three directions, unlabeled spots, and fronts as in *A*. After three directions the polar lipid lane was isolated before rechromatography in the second direction using CMW as the developing solvent.

Other procedures have been devised for the separation of lipid mixtures with a wide range of polarity. The one-dimensional "gradient elution" technique of Tarr (14) is sensitive to adsorbent mesh size and layer thickness, and apparently requires a 5°C development temperature for optional resolution of neutral lipids.

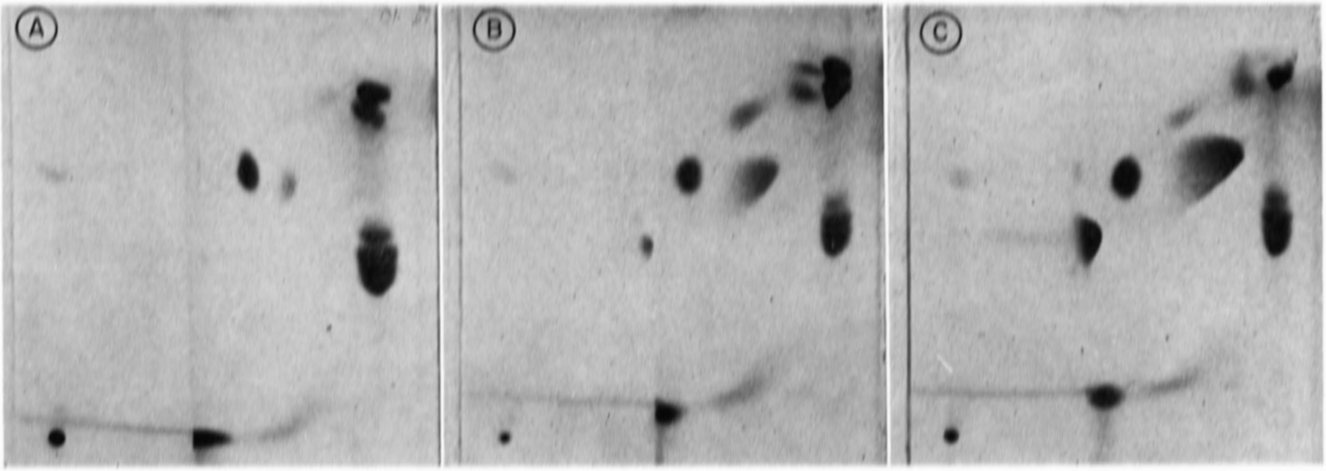


FIG. 6. Thin-layer chromatograms of 1 mg of human serum lipids by four-directional-development TLC. All other conditions as for Fig. 5, *B*. *A*, normal subject; *B*, patient with type IV hyperlipidemia; *C*, patient with type V hyperlipidemia, father of patient *B*.

Tarr's procedure does not resolve 1- and 2-monoglycerides, nor does it claim to be without diglyceride isomerization. The one-dimensional polyzonal procedure of Niederwieser (15) and the two-step development system of Skipski and coworkers (2, 4) do not appear to resolve isomeric mono- or diglycerides.

Isomeric monoglycerides and diglycerides have been resolved using boric acid-impregnated plates (16). However, this technique has not been applied to the simultaneous resolution of other lipids such as glycolipids, cholesterol, methyl or cholesteryl esterified fatty acids, and less polar lipids. The resolution of 1- and 2-monoglycerides, with isomerization, has also been accomplished using hydroxylapatite adsorbent (17).

Our four-directional TLC procedure does not require a special adsorbent, low-temperature development, or impregnation. It also prevents isomerization of 1,2- and 1,3-diglycerides, and presumably 1- and 2-monoglycerides, during their resolution from each other and from a variety of other lipids.

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