The alk-1-enyl group content of mammalian myelin phosphoglycerides by quantitative two-dimensional thin-layer chromatography

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ABSTRACT   Myelin phospholipids have been examined by a separation–reaction–separation procedure for two-dimensional thin-layer chromatography on silica gel. After separation in one dimension, alk-1-enyl groups are cleaved by exposure of the plates to HCl fumes. Development in the second dimension quantitatively separates acid-labile and acid-stable phosphoglycerides as well as the aldehydes released from the acid-labile phosphoglycerides.

Myelin phospholipids from the central nervous systems of the rhesus monkey, squirrel monkey, ox, and mouse contain 32–36% acid-labile ethanolamine phosphoglycerides (ethanolamine plasmalogens) and 8–14% acid-stable ethanolamine phosphoglycerides. Acid-labile choline and serine phosphoglycerides account for less than 1% of the myelin phospholipids.

KEY WORDS   alk-1-enyl groups · plasmalogens · myelin lipids · ethanolamine · choline · phosphoglycerides · thin-layer chromatography

THE PHOSPHOGLYCERIDES that contain alk-1-enyl groups (plasmalogens) account for a substantial portion of the phospholipids of mammalian brain (1, 2) and heart (3). Monolayers of 1-alk-1′-enyl 2-acyl phosphoglycerides have physical properties quite different from those of the corresponding diacyl compounds (4, 5), but the function of plasmalogens in membranes, if any, is unknown (1). According to many reports (6–15), plasmalogens account for 31–36% of the phospholipid content of mammalian central nervous system myelin. In some cases, higher (9, 16, 17) or lower (9, 18–21) values have been given. Most of the brain plasmalogens are ethanolamine phosphoglycerides (2). Specifically, in bovine myelin (6) 35% of the phospholipid molecules and 70% of the EPG contain alk-1-enyl groups while less than 5% of the choline and serine phosphoglycerides contain alk-1-enyl groups. With different methods for plasmalogen assay, O'Brien and Sampson have found SPG from human myelin to contain a considerable proportion of alk-1-enyl groups (17).

Two approaches have been available for the estimation of the alk-1-enyl group content of specific phosphoglycerides. One approach is to separate the desired phosphoglycerides by column chromatography and to estimate the alk-1-enyl group content by iodine addition (22), by preparation of an aldehyde derivative (17, 23), or by differential hydrolysis (24). The other approach requires mild alkaline and acid hydrolysis of the mixed lipids followed by paper-chromatographic separation of the water-soluble moieties (25, 26). Both methods are time-consuming.

A separation–reaction–separation two-dimensional TLC method for the separation of diacyl phosphoglycerides from the corresponding acyl alk-1-enyl phosphoglycerides was described recently by Owens (27). Alk-1-enyl groups were hydrolyzed by a $HgCl_2$ spray reagent. Schmid and Mangold (28) have reported that HCl fumes quantitatively hydrolyze alk-1-enyl groups from neutral glycerides. In the present investigation, a similar two-dimensional TLC method with cleavage of phosphoglyceride alk-1-enyl groups by HCl has been used for the determination of the alk-1-enyl content of myelin phosphoglycerides from four mammalian species. A preliminary report has appeared (29).

MATERIALS AND METHODS

Myelin Preparations

Bovine tissues were obtained from the slaughterhouse within 30 min of death and stored on ice for less than 1 hr before processing was begun. Rhesus monkey tissues were obtained when the animals were 9 months old and were frozen for less than 1 month before processing. Squirrel monkey spinal cords (14) and mouse brains (15) were weighed and dispersed immediately. The mice (strain C57BL./10) were 41–47 days old.

Details of the myelin isolation procedures have been described (14, 15). Crude mitochondrial fractions were suspended in 0.8 M sucrose and centrifuged. The floating layer, after osmotic shock, was purified by flotation (murine myelin) or density-gradient centrifugation (bovine and simian myelin). The mouse microsomal fraction included the material that sedimented from 0.32 M sucrose between 2.0×10^5 and 6.3×10^6 g-min (15). Lipid extracts were prepared (14, 15) from mixtures of $CHCl_3$–CH_3OH 2:1 and aqueous suspensions of the myelin or microsomal fractions.

Lipid Assays

For the determination of phosphorus content, the method of Bartlett (30) was used for lipid extracts and the method of Gottfried (31) for areas scraped from thin-layer plates and for fractions from hydrolysis experiments. The alk-1-enyl group content of lipid extracts was determined by the iodine addition method of Gottfried and Rapport (22) and by mild alkaline hydrolysis followed by mild acid hydrolysis, as described previously (24), using the hydrolysis schemes of Pries, Aumont, and Böttcher (32) and of Dawson, Hemington, and Davenport (25) as modified by Ansell and Spanner (26).

Thin-Layer Chromatography

Thin-layer plates were coated with a 0.5 mm layer of Silica Gel G suspended in 0.01 M Na_2CO_3. After the plates had been activated at 110°C for 30 min a sample containing 0.2–0.4 μmole of lipid P was applied as a series of spots on a 2 cm line in the lower left corner, 2 cm from each side of the plate. The plate was developed in an unlined tank for 12 cm with a mixture of 65 ml of $CHCl_3$, 25 ml of CH_3OH, and 4 ml of 15 N NH_4OH (33). The plate was removed and dried in a stream of ambient air for 10–15 min. The silica gel layer was exposed to the fumes from 12 N HCl at a distance of 4.5 cm from a layer of acid in a Pyrex tray for 10 min. The plate was dried again for 15 min, then developed from the left edge for 10 cm with a mixture of 100 ml of $CHCl_3$, 50 ml of CH_3OH, and 12 ml of 15 N NH_4OH. After the plate had dried, spots were located with iodine vapor (e.g., Fig. 1) and marked with a stylus. The areas were
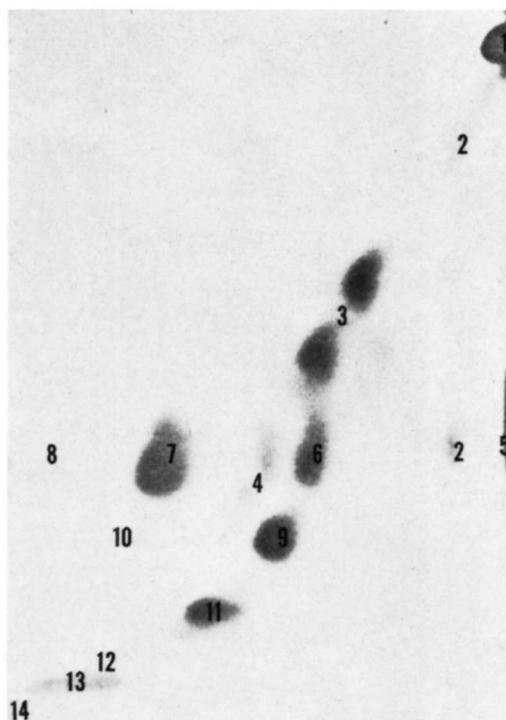

Fig. 1. Chromatogram of ox optic nerve myelin lipids, applied at spot 14. First development vertically with chloroform–methanol–ammonia 65:25:4. After exposure to HCl fumes, second development to the right with chloroform–methanol–ammonia 100:50:12. Iodine stain. The areas are: 1, cholesterol; 2, unidentified; 3, cerebrosides; 4, cerebroside sulfates; 5, aldehydes released from acid-labile EPG; 6, acid-stable EPG; 7, monoacyl GPE; 8, GPE; 9, acid-stable CPG; 10, monoacyl GPC; 11, sphingomyelin; 12, inositol phosphoglycerides; 13, serine phosphoglycerides; 14, origin.

removed by scraping with a flexible plastic ruler (2.5 × 15 cm) onto weighing paper.

EPG preparations were made by preparative TLC (34). The Silica Gel G with the EPG was applied to a column packed with 1 g of Unisil (activated silicic acid, Clarkson Chemical Company, Inc., Williamsport, Pa.) slurried in $CHCl_3$. The EPG was eluted with 50 ml of $CHCl_3$–CH_3OH 2:1.

For identification of the nonpolar cleavage products, TLC plates were developed in one direction only with a mixture of 90 ml of hexane and 10 ml of diethyl ether.

RESULTS

Identification of HCl Cleavage Products

Bovine brain phospholipid and EPG preparations were applied to thin-layer plates and exposed to HCl fumes. To the same plate we then applied material eluted from area 5 (Fig. 1) of another plate together with authentic samples of oleic acid, methyl oleate, myristaldehyde, and the dimethyl acetal of myristaldehyde. After development with a mixture of hexane and diethyl ether,

the plate was stained with iodine. The only detectable spot of phospholipid origin had a mobility identical with that of myristaldehyde.

Phospholipid spots were identified after two-dimensional development as described previously (33). The material in area 7 (Fig. 1) had a mobility in the second dimension corresponding to that of monoacyl GPE. Insignificant amounts of phosphorus were found in area 8, which would contain any GPE derived from the EPG. Area 10, monoacyl CPG, accounted for 1–2% of the total CPG in ox and monkey myelin, but was present in only trace quantities in mouse myelin. Very small amounts of material were found at the second-dimension solvent front above the sphingomyelins and SPG (far right of Fig. 1). The acid-labile SPG are not clearly separated by this procedure. The recovery of phosphorus from 40 consecutive experiments was 90.8 ± 3.1% (mean ± SEM).

Completeness of Alk-1-enyl Group Cleavage

The relative amount of phosphorus in the monoacyl EPG can be compared in Table 1 with the alk-1-enyl group content of the total lipids as determined by the iodine addition reaction. The ratios of alk-1-enyl group:phosphorus for the lipids of ox optic nerve myelin determined by two sequential hydrolysis methods (24, 32) were 0.354 and 0.316, respectively. Norton and Autilio have reported values of 0.348 (6) and 0.320 (8) for ox brain myelin.

DISCUSSION

Two-Dimensional TLC

All of the evidence indicates that the cleavage of alk-1-enyl groups was quantitative. For ox myelin, the results obtained by four different methods were in excellent agreement with those of Norton and Autilio (6, 8). For other lipid extracts, the results from the TLC procedure agreed with those from the iodine addition method. In each case, the variance was smaller for the TLC procedure. The brain lipid extracts contained only very small amounts of alk-1-enyl groups in classes other than the EPG. The absence of significant amounts of phosphorus in area 8 (Fig. 1) indicates that the EPG were not degraded during the drying process and that significant quantities of the dialk-1-enyl GPE do not occur in mammalian myelin. The recovery of phosphorus from the plates was over 90%, which is slightly less than with one-dimensional TLC (31). The lower recovery may have been due to the omission of small spots and losses during the scraping process. Any lack of uniformity in scraping would be reflected in the standard errors.

The separation–reaction–separation procedure for two-dimensional TLC of phospholipids is similar to that of Owens (27) but uses a reaction that is easier to perform and does not require highly toxic chemicals. The use of HCl for cleavage of the alk-1-enyl ether bonds of glycerides was described earlier by Schmid and Mangold (28). Recent, alternative approaches for the measurement of the alk-1-enyl group content of the EPG include one-dimensional TLC after alk-1-enyl group cleavage with 1.2 N HCl–methanol in a portion of the extracts (13). Alk-1-enyl group contents are calculated by difference instead of directly. Another approach is to isolate a specific phosphoglyceride class, subject it to alk-1-enyl group cleavage, and examine the products by TLC (24, 35, 36). Unfortunately it is quite difficult to isolate pure phospholipids with complete recovery of the alk-1-enyl components (24). In some instances, intact EPG plasmalogens have been recovered in the SPG fractions from DEAE-cellulose columns (L. A. Horrocks, unpublished data). This observation may partially explain the rather high content of alk-1-enyl groups in SPG fractions from DEAE-cellulose separations of human brain myelin lipids (17).

The present method seems to be the simplest and most reliable one for the determination of the alk-1-enyl group content of specific phosphoglyceride classes and should be of value for the study of the incorporation of radio-

TABLE 1   Determination of Acid-Labile (Plasmalogen) and Acid-Stable Ethanolamine Phosphoglycerides of Mammalian Central Nervous System Myelin and Microsomal Lipids

Species, Tissue, Fraction	n	Thin-Layer Chromatography		Proportion of Plasmalogens by Iodine Addition (22)
		Acid-Stable EPG	Monoacyl EPG	
		mole fraction by P determination		
Mouse, brain, microsomes	16	0.171 ± 0.004	0.163 ± 0.003	0.157 ± 0.007
Mouse, brain, myelin	18	0.140 ± 0.004	0.326 ± 0.006	0.334 ± 0.009
Ox, optic nerve, myelin	1	0.077	0.333	0.328
Ox, spinal cord, myelin	1	0.128	0.334	0.333
Squirrel monkey, spinal cord, myelin		0.089 ± 0.004	0.356 ± 0.009	0.374 ± 0.017
Rhesus monkey, medulla oblongata, myelin	8	0.091 ± 0.003	0.354 ± 0.003	—
Rhesus monkey, corpus callosum, myelin	8	0.085 ± 0.004	0.336 ± 0.007	—

active compounds into complex phospholipid mixtures containing alk-1-enyl groups. In contrast to earlier methods (37), the present method permits the study of the nonpolar groups as well as the water-soluble moieties such as glycerol and ethanolamine. The aldehydes released from each specific class of phosphoglyceride are found in separate areas of the plate.

Myelin Composition

The acid-stable EPG content of central nervous system myelin phospholipids is less than 15% of the total phospholipid and is species-dependent (Table 1). The acid-stable EPG could include diacyl GPE, acyl alkyl GPE, and dialkyl GPE. The two former types are known components of mammalian brain and some evidence for the existence of the latter type has been reported (24, 35). Preliminary experiments have shown that alkyl GPE compounds can be isolated from the acid-stable EPG mixture after methanolysis (29).

It is obvious that the proportion of central nervous system myelin phospholipids found in the acid-labile EPG fraction is very similar in each of the four mammalian species, which include two primate species. The microsomal lipids from mouse brain have a much lower proportion of acid-labile EPG. The acid-labile EPG could include acyl alk-1-enyl GPE and alk-1-enyl alkyl GPE, but no evidence for the existence of the latter type has been reported.

By consideration of the present results and reports in the literature on the phospholipids from mammalian central nervous system myelin, it seems quite likely that the correct values for the mole fraction of acyl alk-1-enyl GPE are in the range of 0.31–0.36. Choline and serine plasmalogens are present in very small amounts. Higher values can be attributed to defective methods for determination of the alk-1-enyl group content. Lower values can also be caused by dilution of the myelin preparation with other subcellular components or by accidental partial hydrolysis of the acyl alk-1-enyl GPE.

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