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THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF MYELIN LIPIDS, THEIR DIFFERENTIAL O-DEACYLATION BY PRIMARY ALKYLAMINES AND THEIR SELECTIVE STAINING BY THIONINE

A LIMITED PHYLOGENETIC STUDY[a]

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SUMMARY

A silica gel thin-layer chromatographic procedure is described for the study of the myelin lipid patterns in a small phylogenetic series of nerve tissue specimens. It involves the selective staining by the thiazine dye thionine and the interpretations were facilitated by a preceding primary alkylamine O-deacylation step. Glycolipids, including sulfatides, and ethanolamine plasmalogens were the principal characterizing lipids.

INTRODUCTION

There is a general recognition [1] that the myelin sheath of nerve fibers is characterized by a relatively high content of a number of glycolipids, i.e., galactosyl diglyceride (GDG), normal fatty acid and hydroxy fatty acid ceramide monohexosides (n-CMH and h-CMH, respectively) and ceramide monohexoside sulfatides (CMH-S). In addition, a molecular species (PE_2) of ethanol-

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amine plasmologen (alkenyl glyceryl ethers) has been shown to correlate with the myelination process [2]. Less ubiquitously involved is the N-acyl derivative of ethanolamine phospholipids (NAPE) including an alkenyl analogue [3]. A relationship between various minor O-acylated CMH [4] to myelin has not yet been established.

The empirical selection of the thiazine dye thionine (Lauth's violet) for the metachromatic detection of sulfatides on thin-layer chromatograms led to observations of staining behavior related to (a) the alkenyl lipids, (b) metachromasia[a] of the acidic phospholipids cardiolipin (CL), phosphatidylglycerol (PG), bis-phosphatidic acid (bis-PA), phosphatidylserine (PS) and phosphatidylinositol (PI) and (c) glycolipid detection via periodic acid oxidation. Since thionine has two free amino groups, we had perceived it to be a potential Schiff's base reagent and it was, therefore, used here for the detection of aldehydes derived from (a) plasmalogens by $HgCl_2$ or by HCl and (b) from glycolipids following periodic acid oxidation.

From these observations, we have developed a program of thin-layer chromatographic (TLC) analysis sufficient to account for all of these lipids. In large part, the co-chromatography problems encountered were eliminated by the mild and efficient O-deacylation by monomethyl-or monoethylamines, as initially shown for neutral lipids of other tissues [7]. A limited phylogenetic approach was taken in the selection of nerve tissue specimens for TLC examination.

EXPERIMENTAL

Nerve cord, entire brain, spinal cord and optic nerve were, appropriately, taken from fresh specimens of an invertebrate, a cyclostome, various cartilaginous and bony fish and an amphibian[b], and promptly frozen and lyophilized prior to extraction with chloroform–methanol (2:1) at a ratio of 5 ml per 100 mg dry weight of tissue. The general TLC procedures employed have been discussed earlier [8] for 10 cm×10 cm Polygram Sil G (Macherey-Nagel, Düren, F.R.G.) plastic backed sheets (freshly extracted, sequentially, with chloroform–methanol and by acetone) where 10 μl of the above extracts provided adequate sample for the TLC analysis. As needed, standard lipid samples were used (Serdary Research Labs., London, Canada). Baker-Flex IB2 (Phillipsburg, NJ, U.S.A.) and Whatman PE Sil G (Maidstone, U.K.) were also found to be usable; observations on Macherey-Nagel Sil N-HR will be reported.

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[a]Metachromasia has a long history of observation, perception and explanation [5]; there has been little application to lipids [6].

[b]The squid *Loligo* (a cephalopod mollusc), the sea lamprey *Petromyzon*, the shark *Alopias*, the teleost fish *Cynoscion nebulosis* (sea trout), *Leiostomas xanthurus* (spot), *Pogonia cromis* (drum), *Amia calva* (bowfin) and the salamander *Necturus* (selected as a representative larval amphibian).

The following, empirically determined, mobile phases were used: (A) chloroform–methanol–water (70:24.5:2.7, v/v); (B) chloroform–methanol (9:2, v/v). Addition of 14 *M* NH_4OH, 0.05 ml per 20 ml of mobile phases A and B, respectively, usefully modified the R_F of NAPE and of CL when co-chromatography with CMH or GDG was a problem. Both mobile phase A and B, with or without NH_4OH, can be used in the first dimension of two-dimensional chromatograms with hexane–diethyl ether (4:1, v/v) as the second dimension mobile phase, after the 1% HCl hydrolysis step, for a clear demonstration of alkenyl lipids [8]. Mobile phase C, chloroform–ethyl acetate (3:5, v/v), was used for resolution of the glycerol ethers produced by Vitride reduction [7].

Alkylamine O-deacylation

The reaction is done in 100 m×13 mm screw-capped tubes (PTFE seal) containing 1 ml of extract. After removal of the solvent at 40°C, with a stream of nitrogen, 1 ml of alkylamine[a] is added and the tube is securely capped. The reaction is done at 50°C for 1.5–2 h. For the Fluka reagents, the ethanol and methylamine are removed, as was done for the extract, and the residue is redissolved in chloroform–methanol (2:1, v/v). On the other hand, 1 ml of 1-butanol, as a solvent, is added directly to the aqueous alkylamine reaction mixtures followed by 4.5 ml of butanol-saturated 0.85% NaCl; vigorous mixing transfers the lipids to the butanol upper phase. The ethylamine is required if the N-methyl fatty acid amides, resulting from methylamine O-deacylation, present a co-chromatography problem as occurs when chloroform–ethyl acetate (3:5, v/v) is used as mobile phase.

Spot-testing

The thionine reaction solution consists of 3 mg of dye per 100 ml of water; staining is for 7–10 min followed by repeated washing with 0.05 *M* H_2SO_3 to remove the violet background stain. The chromatogram is then blotted with paper toweling and air-dried; it is best observed by transmitted light. Leucothionine is prepared by adding 20 ml of 1 *M* H_2SO_3 to 20 mg of dye in 50 ml of water; the reduction requires several hours. A longer staining time than for thionine is required, e.g., 30 min, followed by repeated washing in 0.05 *M* H_2SO_3. We will comment later on our experience with other thiazine (and oxazine) dyes. The Camag (Muttenz, Switzerland) TLC densitometer II, reflectance mode, was used to determine the absorption spectra of the variously stained lipids. Spingomyelin, the diacylphosphatidyl cholines and the phosphatidyl ethanolamines are distinctively visualized by the Biebrich Scarlet spot-test [8], λ_{max} 520 nm, readily differentiated from the thionine-staining components.

[a]Methylamine (Fluka Chemie, Buchs, Switzerland or Aldrich, Milwaukee, WI, U.S.A.), 33% in ethanol and 40% in water, respectively, or ethylamine (Aldrich), 70% in water.

RESULTS

Methodology observations

Figs. 1 and 2 show the indicated resolution of the polar lipids where the plasmalogens (alkenyl) are clearly characterized by staining green with thionine (our visual perception) with a λ_{max} at ~625 nm (Fig. 3A), while sulfatides (CMH-S) and the acidic phospholipid CL stain violet (λ_{max} ~600 nm, Fig. 3B); like CL, all of the acidic phospholipids however, can stain violet. Thionine staining, therefore, serves to unequivocally differentiate bis-PA from NAPE [9]. The TLC-resolved alkenylglycerol, resulting from Vitride reduction of plasmalogens [8], stains green with thionine while alkylglycerol (from 1-alkyl-2-acylphosphoglycerides) remains unstained unless there was a prior periodic acid oxidation, in which case it stains violet, representing, therefore, an alternative to the leucofuchsin plasmal reaction [8]. The initial color response of GDC and CMH following periodic acid oxidation is violet, which may be followed by a darkening transition to blue–green (with a narrow violet halo) as the H_2SO_3 washing process is continued. This may be reflected in the shape

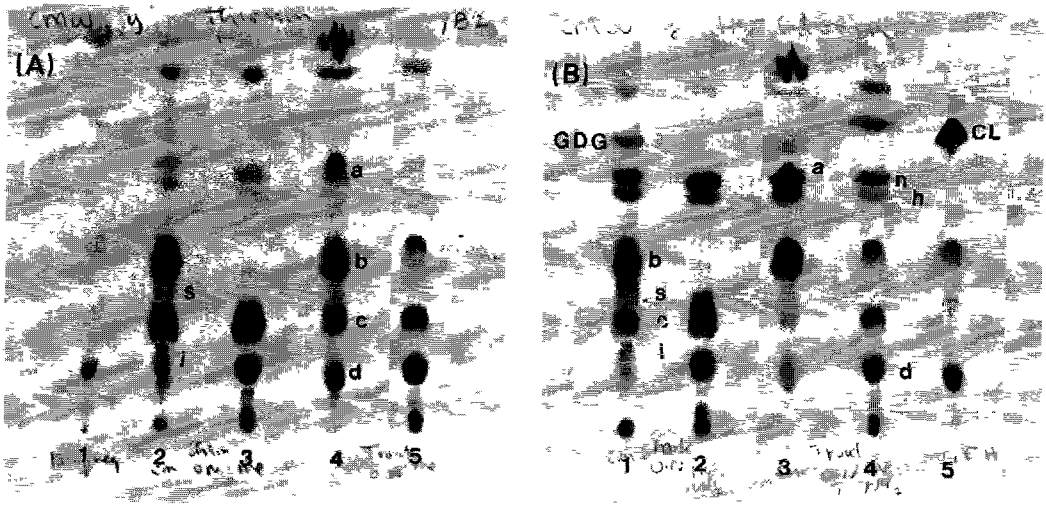

Fig 1 (A) Thin-layer chromatogram showing the thionine staining of nerve tissue lipids in various animals. Lanes 1=lamprey nerve cord; 2=shark optic nerve (chloroform–methanol control), 3=methylamine products, 4=trout optic nerve control; 5=methylamine products. The mobile phase is chloroform–methanol–water (140 49 5 5, v/v) and the stationary phase is Baker IB2. a=NAPE (trout) co-chromatographing with CMH; b=PE; c=mainly sulfatide (CMH-S); d=lyso-PE (as alkenyl in the methylamine samples and PC in controls); s=PS; i=PI. (B) Chromatogram similar to (A) except that a periodic acid oxidation step preceded the thionine staining; the lipids, however, are similarly indicated. The glycolipids are visualized by this procedure. GDG=galactosyl diglyceride; n=n-CMH; h=h-CMH, CL=cardiolipin (stains directly with thionine as discussed in the text). Lanes 1–4 as for (A), 5=guinea pig heart.

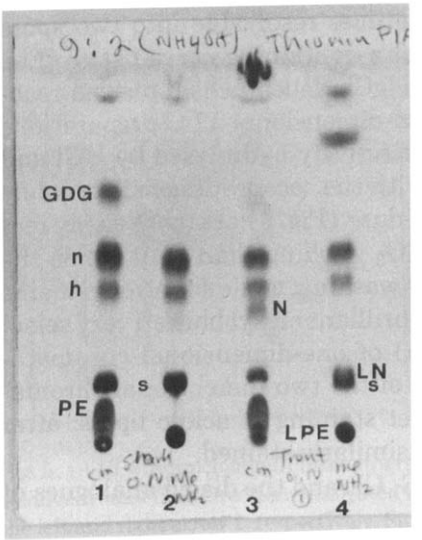

Fig 2. Chromatogram demonstrating the usefulness of chloroform–methanol–ammonia (see text) in resolving the characterizing lipids of nerve tissue. The chromatogram was stained first with thionine, for initial observations, followed by periodic acid oxidation and a second staining with thionine (as shown here). Lanes: 1 = shark optic nerve control; 2 = methylamine reaction product; 3 = trout optic nerve control, 4 = methylamine reaction product. Labeling similar to Fig. 1; s = CMH-S; N = NAPE; LN = lyso-NAPE.

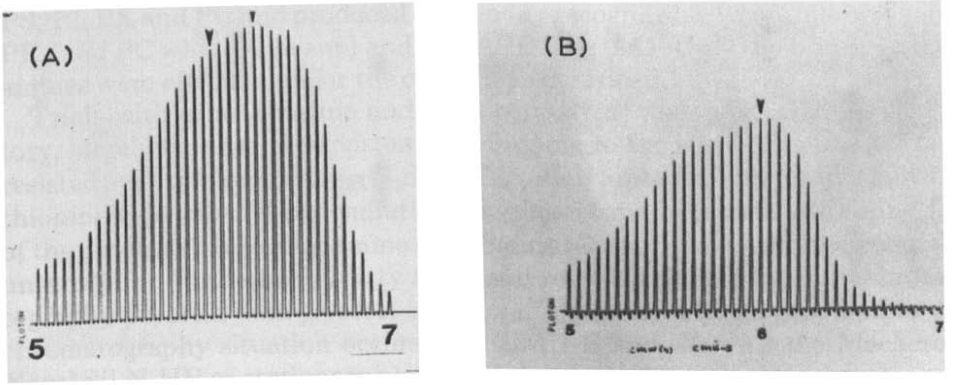

Fig. 3. (A) Absorption spectrum of the ethanolamine plasmologen–thionine complex by direct scanning of a chromatogram from 500 to 700 nm in 5-nm increments. The large arrow is at 600 nm. (B) Absorption spectrum of the sulfatide–thionine complex from a TLC preparation.

of the curve of its absorption spectrum (Fig. 3B) as representing two substances, not seen when leucothionine is used since no violet staining occurs. Non-alkenyl lipids have a generally low (and not very useful) affinity for thionine. The largely alkenyl phosphatidyl choline of guinea pig heart (Fig.

4B) is consequently easily recognized, sometimes resolvable into two spots [10], as well as do both PE_1 and PE_2 of myelin-containing nerve tissue (Fig. 4A), although shown here as stained by the $HgCl_2$–leucofuchsin plasma reaction. Both are more easily resolved in the two-dimensional TLC preparations (Fig. 4B) where the alkenyl ether bond is selectively hydrolyzed by HCl and the resulting aldehydes selectivity migrate with the second-dimension mobile phase. Free aldehydes produced by this procedure (Fig. 5) are more easily recognized as green in the initial phase of H_2SO_3 washing than they are as the optical density increases with more extensive washing, while sulfatide remains violet. Leucothionine, leucoazure c and leucobrilliant cresyl blue all very selectively stain the alkenyl components (green) of one-dimensional chromatograms and of the alkenyl-derived aldehydes of the two-dimensional chromatograms, nearly totally represssing the violet staining of acidic lipids; after periodate oxidation CMH and CDG become similarly stained.

The alkylamines clearly removed the GDG, CL and the diacyl analogues of

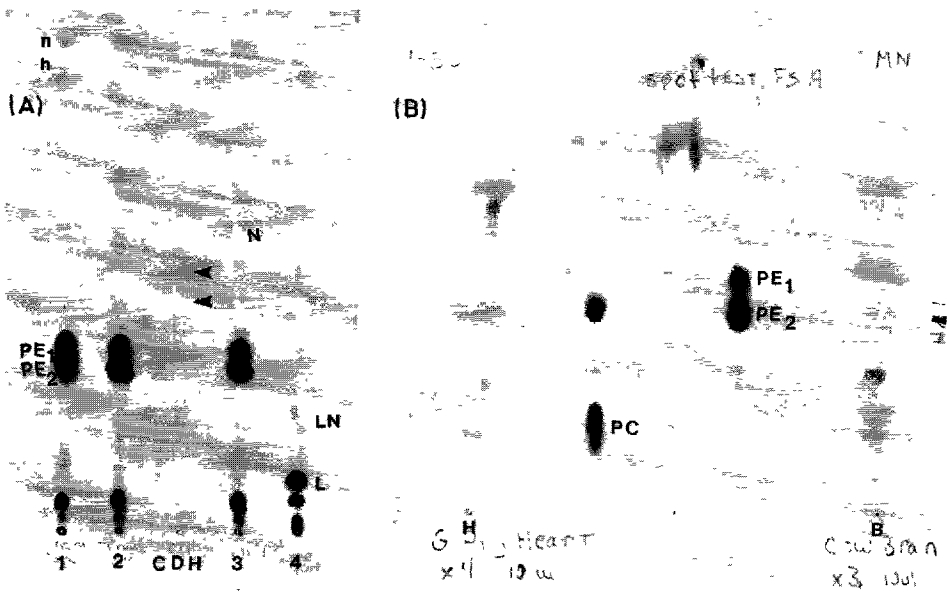

Fig. 4 (A) Resolution of the two PE plasmalogens is shown on this 10 cm × 20 cm chromatogram with chloroform–ethanol–water (65.25 2.5, v/v) as mobile phase The plasmalogens were visualized by the $HgCl_2$-catalyzed leucofuchsin reaction, with Biebrich-Scarlet as counter stain. Labeling is as indicated for n- and h-CMH and intact and lyso-NAPE. The resolution of PE plasmalogens is indicated as PE_1 and PE_2. The ceramide dihexoside standard is indicated by the two arrows. Lanes: 1 = shark optic nerve; 2 = trout brain; 3 = *Spot* brain (control); 4 = methylamine. (B) Two-dimensional thin-layer chromatogram as for Fig 5A and B. Guinea pig heart (left) and cow optic nerve (right) leucofuchsin spot-test. The resolution of heart PC and of optic nerve PE (cf A), seen here as alkenyl-derived aldehydes, is shown. These two-dimensional chromatograms have been referred to as $2D_{HCl}$ in ref. 8.

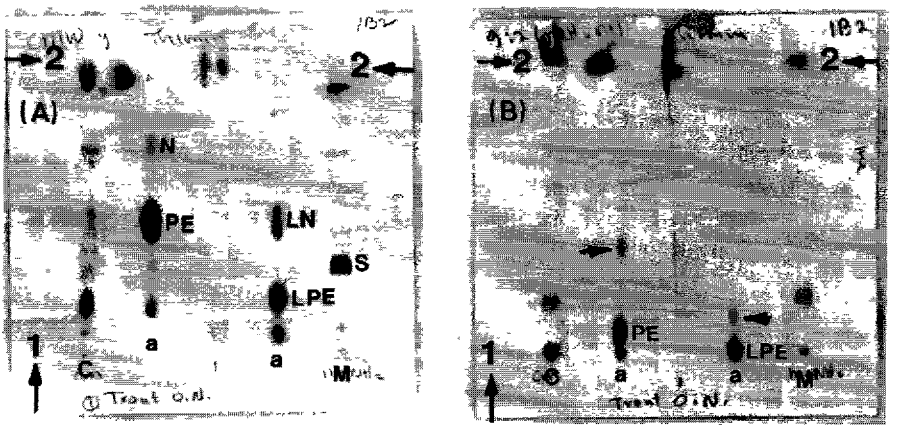

Fig. 5. (A) Two-dimensional chromatogram with chloroform–methanol–water as mobile phase in a conventional first direction (indicated by arrow 1). The second dimension(s) are indicated by the left and right arrow 2, respectively. Sample application was at the origin of the two outer lanes (C = control; M = methylamine) while the free aldehydes, released from the various alkenyl phospholipids by the 1% HCl step (see text), migrate with the second-dimension mobile phase (hexane–diethyl ether, 4 1) as seen in the two respective inner lanes (a) following thionine staining. Trout optic nerve control at left (C) and methylamine O-deacylation (M) at right; N and LN are from NAPE and lyso-NAPE, respectively; S is CMH-S. (B) Same as for (A) except that the first dimension is chloroform–methanol (9 2, v/v) with added NH_4OH as for Fig 2; here the upper and lower arrows represent the aldehydes derived from NAPE and lyso-NAPE, respectively.

PS, PI, PE and PC and produced the readily recognizable lysoforms of alkenyl PE (and PC where relevant) and of NAPE (Fig. 1A). Only the primary alkylamines were effective under the conditions described.

Trials with other thiazine and of oxazine dyes[a] were generally unsatisfactory, largely because of persistent dye-binding to the stationary phase which resisted selective removal by H_2SO_3. They did, however, frequently show the thionine-type alkenyl and sulfatide staining. Osmium tetroxide staining [8] of the products of the alkylamine and Vitride reactions uniformly reflected the unsaturation in the initial fatty acids and revealed the potential co-chromatography problem with glycerol ethers and its solution (Fig. 6). Another co-chromatography situation occurs with CMH-S and CL with the Machery-Nagel Sil N-HR as stationary phase and the chloroform–methanol–water mobile phase with suppression of violet staining. We have had limited experience with silica gel on glass plates but it has been sufficient to implicate the influence of various bonding materials (and perhaps of integral fluorescence indicators) on the staining response to thionine, as well as to specific R_F changes with chloroform–methanol–water, notably with CL (cf. Fig. 1B) which can co-chromatograph with PE, i.e., Analtech silica gel GHL (with inorganic binder),

[a]Azure A, B, C, methylene blue, toluidine blue; cresyl violet and brilliant cresyl blue.

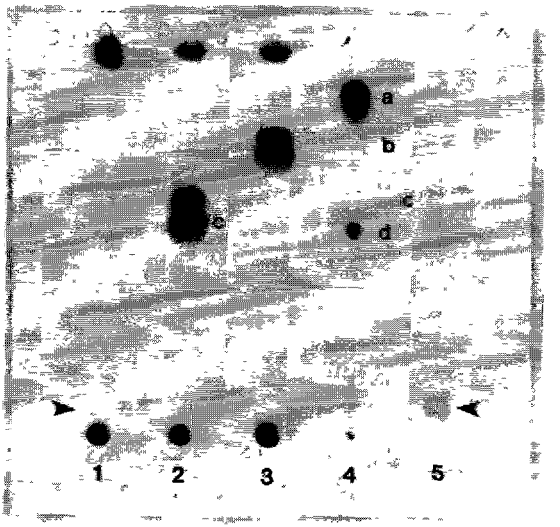

Fig. 6. Chromatogram showing the N-alkyl fatty acid amides as resolved from the long-chain aldehydes produced from the released fatty acids by Vitride reduction (see text). The mobile phase was chloroform–ethyl acetate (3:5, v/v) with OsO_4 vapor as the spot-test. Sample: *Amia* (fish) brain. Lanes: 1 = control; 2 = methylamine; 3 = ethylamine; 4 = Vitride; 5 = GDG standard. Vapor-phase OsO_4 staining. a = Fatty alcohols; b = N-ethyl fatty acid amides; c = N-methyl fatty acid amides; d = alkenyl glycerol. The arrows indicate the position of GDG.

which is also adversely sensitive to periodic acid oxidation (Analtech, Newark, DE, U.S.A.), or can co-chromatograph with CMH-S, i.e., with HPTLC silica gel 60 F254 (E. Merck, Darmstadt, F.R.G.). Finally, it should now be apparent that, for thionine, we cannot properly be referring to violet staining as metachromatic since the aqueous color of the dye itself is violet as is the dye when chromatographed on a silica gel stationary phase (each with a $\lambda_{max} \sim 600$ nm).

Phylogenetic observations

(1) Of the specimens examined, only squid and lamprey nerve cord are considered to be unmyelinated, as compared to the shark to *Necturus* sequence. Their TLC patterns were simple and quantitatively low, they had none of the myelin lipids referred to above, and their alkyl and alkenyl content was correspondently low, as determined by the Vitride reduction TLC technique, with the alkyl content clearly greater than the alkenyl.

(2) The myelin lipid content of the shark specimens was generally greater than that in the teleost fish and *Necturus* (Fig. 1) and, indeed, was particularly distinguished by the high sulfatide content, not matched by a similar CMH content (Figs. 1B and 2).

(3) The teleost fish provided the only specimens where NAPE could be demonstrated (Figs. 1 and 2), best seen as the alkenyl analogue, as demonstrated

by the free aldehyde of two-dimensional chromatograms (Fig. 5) both as intact phospholipid and as the alkylamine-produced lyso derivative.

(4) *Necturus* had a typical adult mammalian pattern.

(5) No evidence of O-acylated CMH [5] was detected, i.e., as an increase in CMH following alkylamine O-deacylation. CL (as a mitochondrial marker) could sensitively be detected because of its shape and thionine color even when co-chromatographing with CMH (Fig. 1B).

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