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Note

Thin-layer chromatographic resolution of molecular species of ethanolamine plasmalogen quantitatively unique to myelin

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Ethanolamine plasmalogen (1-alk-1′-enyl,2-acyl-*sn*-glycero-3-phosphoryl-ethanolamine) is widely recognized as a major nerve tissue lipid occurring in both grey and white matter of brain. The first clear evidence of its presence in the myelin sheath of nerve fibers evidently came from the histochemical studies of Verne[1], in 1928, in an application of the Feulgen and Voit[2] Schiff's reaction for plasmalogen. We can abundantly confirm this observation, both for the central nervous system and the peripheral nervous system, from our own histochemical experience[3]; *cf.* Fig. 1a–c. The chemical structure, however, of ethanolamine plasmalogen was not worked out until 1954[4]. Final substantiation of the histochemical observations came even later with the analysis of myelin obtained by differential centrifugation procedures which are extensively reviewed by Horrocks[5]. The plasmalogen correlation with the myelination process is now thoroughly recognized although other myelin lipids, *e.g.*, the various sphingolipids, especially cerebrosides, have generally been studied more intensively in this regard.

In the course of recent studies on the plasmalogens of fish brain and optic nerve we observed[6] that plasmalogen staining of thin-layer chromatographic (TLC) plastic sheets (F1500 silica gel; Schleicher & Schuell, Dassel, G.F.R.), developed in the Beiss-2 solvent* mixture, was initially more reactive for the upper portion of the ethanolamine phosphatide (PE) spot than for the lower portion. When the reaction

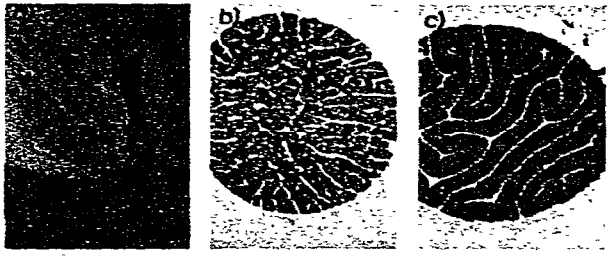

Fig. 1. Fresh frozen cryostat sections, 12 μm thick, stained with the $HgCl_2$–Schiff reagent for plasmalogens. (a) Rabbit cerebellum showing the relatively weak reaction of the cortex as compared to that of the myelinated fiber tracts; (b) cross section of rabbit optic nerve which consists exclusively of myelinated nerve fibers; (c) cross section of optic nerve of the fish *Elops saurus*, showing the intensly stained myelin fibers arranged in a folded laminar fashion commonly seen in fish.

* Tetrahydrofuran–diisobutyl ketone–chloroform–acetic acid–water (45:5:10:6:6).

was complete, however, the entire PE spot appeared to be homogeneously stained. Subsequent chromatographic experimentation successfully demonstrated that this PE spot could be resolved into two ethanolamine plasmalogens of which the lower one (PE-2) appeared to be solely correlated with myelin.

MATERIALS AND METHODS

Fresh, frozen-dried, samples of brain and optic nerve were obtained from several species of mammal (including adult human, new-born and adult mouse and rat), a reptile (the snake *Crotalus atrox*) and several species of fish, spinal cord of rat and guinea pig and sciatic nerve of dog. Chloroform–methanol (2:1) extracts were prepared using 5 ml of solvent per 100 mg dry weight of tissue; several specimens of each were examined except for the snake. The human material was from two males aged 56 and 69 years and was divided into cortical grey matter and corpus callosal white matter. Extracts of various other mammalian, reptile and fish tissues (2 ml of chloroform–methanol per 100 mg of freeze-dried tissue) were also examined for comparison. The general chromatographic and visualizing procedures used have been completely described elsewhere[3,7,8].

Three types of plastic-backed silica gel TLC sheets, without indicator, were employed: (a) Schleicher & Schuell F1500, (b) E. Merck (Darmstadt, G.F.R.) silica gel 60, and (c) Eastman (Rochester, N.Y., U.S.A.) chromatogram sheet 6061. These were all generally used without a pre-wash with chloroform–methanol and acetone to clean up the solvent front area. Recognition and characterization of the plasmalogens required various *in situ* reactions with lecithinase A_2, $HgCl_2$ etc. in accordance with our earlier[9] description. Because of the ease of obtaining pure samples of cerebral grey and white matter from adult human brain, the isolates of PE were prepared from these materials using silicic acid columns and TLC plates and standard elution sequences. Fluram (Roche Diagnostics, Nutley, N.J., U.S.A.), in acetone, was used as the amine reagent spray, visualizing the fluorescence at 3660 Å.

RESULTS

The TLC resolution of the two nerve tissue PE plasmalogens (PE-1 and PE-2) was accomplished by the system (chloroform–ethanol–water, 65:25:3) (Fig. 2a–c), although the system (chloroform–methanol–water, 100:12:1) could also be used. Neither added NH_4OH nor added acetic acid modified this resolution of the nerve tissue PE as long as there was compensation in the water content. Both PE spots were positive to the Fluram amine reagent and were essentially completely cleaved by $HgCl_2$ and by the lecithinase A_2 of snake venom (*Ancistrodon piscivorus*), *in situ* application, resulting in complete conversion to the appropriate lyso compounds. Vitride reduction[7,8] revealed that alkyl analogs represented a very small portion of the total PE (Fig. 3). The PE-1 was stained by OsO_4 vapor, consistent with its higher polyunsaturated fatty acid content, as was its $HgCl_2$ lyso product. The two PE substances were not resolved by silicic acid column chromatography by the customary chloroform–methanol elution mixtures. Preparative TLC resolution, however, was easily accomplished by the chloroform–ethanol–water solvent and the PE-1 and

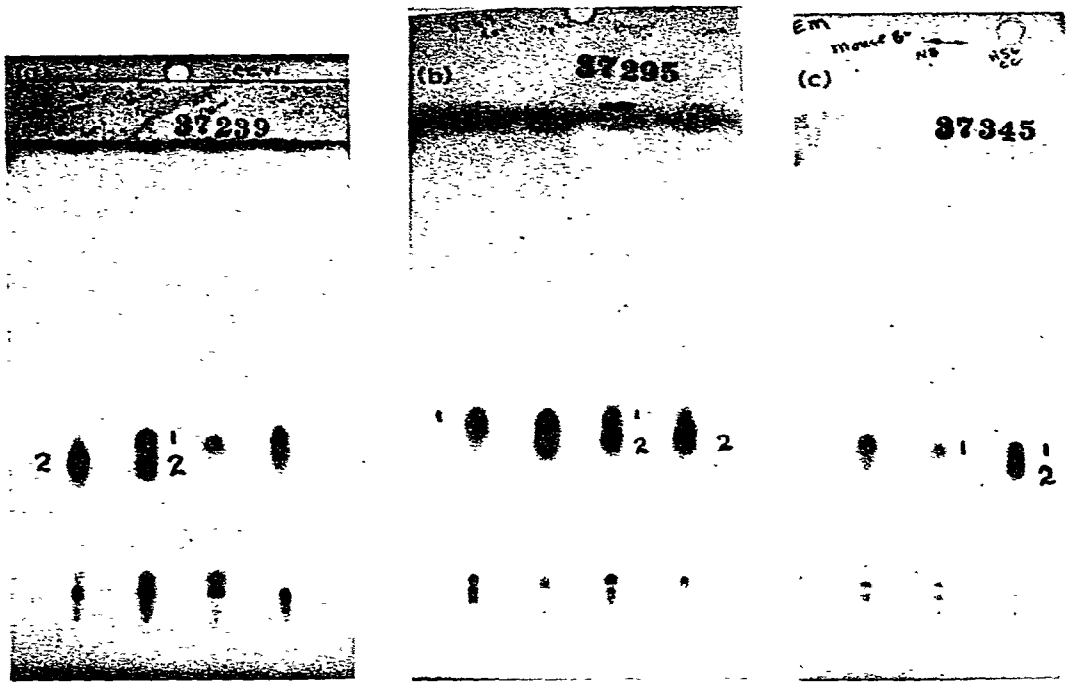

Fig. 2. Silica gel plastic sheets developed in chloroform–ethanol–water and stained, by immersion, in the $HgCl_2$–Schiff plasmalogen reagents. (a) From left to right: optic nerve of fish (*Elops saurus*), brain of shark (*Caraharhinus leucas*), dog pancreas and snake brain (*Crotalus*); (b) from left to right: human (H-69) cerebral cortex and optic nerve, fish (*Amia calva*) brain and optic nerve; (c) from left to right: adult mouse brain, new-born mouse brain, human brain white matter (H-56). PE-1 and PE-2 plasmalogens are indicated as 1 and 2 respectively.

PE-2 (human corpus callosum) separately collected for analysis using Fluram as the indicator (Fig. 4). I_2 was not satisfactory for this purpose because of the resulting increased alk-1-enyl cleavage. Analysis of the acid hydrolysates from both PE-1 and PE-2 revealed ethanolamine as the only amine, substantiating their chromatographic behavior as ethanolamine glycerophosphatides. Preliminary gas chromatographic analysis revealed that 18:1 was the principal fatty acid of the PE-2; polyunsaturated c20 and c22 fatty acids were well represented in the PE-1 and 18:1 was relatively low as compared to its content in PE-2. A major fraction of both PE-1 and PE-2 alk-1-enyl was unsaturated as indicated by bromination (Br_2 vapor) of the *in situ* HCl cleaved aldehydes prior to the chromatographic run (*cf.* Fig. 5).

All whole brain specimens (except the new-born mouse and new-born rat), optic nerve, spinal cord, sciatic nerve and white matter (human brain) contained both of the PE plasmalogens, the PE-2 being the dominant one in all specimens not containing cortical tissue (*cf.* Fig. 2). Human cerebral cortex, when carefully collected to exclude the underlying white matter, contained only PE-1 plasmalogen as did all of the non-nervous tissue specimens listed. Relatively little diacyl PE analog accompanied the nerve tissue PE in marked contrast to the other tissues whose alkyl-acyl PE content was also, generally, higher.

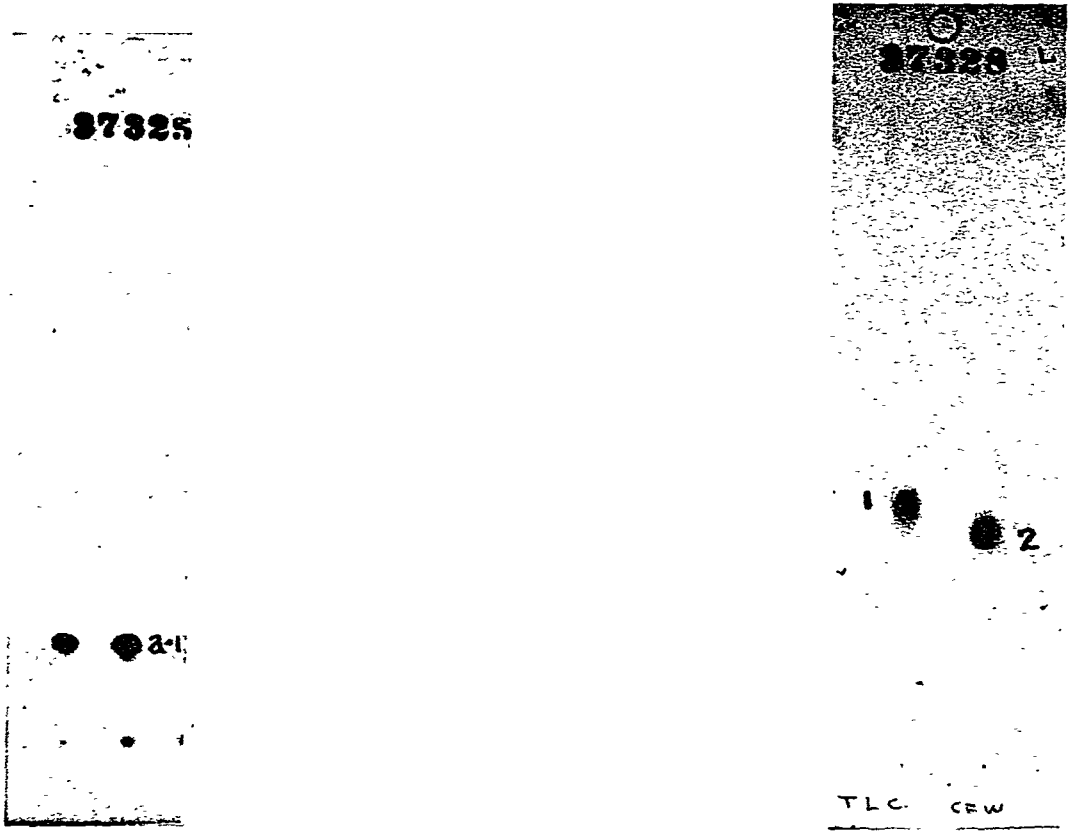

Fig. 3. Glyceryl ethers derived from Vitride reduction of human cerebral cortex (left) and corpus callosum (right) showing the dominance of alk-1-enyl (a-1) in both samples.

Fig. 4. Schleicher & Schuell F1500 chromatograms of human brain white matter isolates of PE-1 (left) and PE-2 (right) developed in chloroform–ethanol–water, plasmalogen stain.

Of the three types of TLC sheets referred to above only F1500 (Schleicher & Schuell) was fully satisfactory for our purpose because of its better wetting properties and resistance to washing. Glass plates could not be used with any of the dipping procedures we use for spot-testing because of the ease with which the media comes off the plate when wetted in this manner.

From these observations and in consideration of the data of O'Brien *et al.*[10–12] and Sun *et al.*[13,14] it is our presumption that the PE-2 plasmalogen of nerve tissue described here, revealed by silica gel TLC developed in the chloroform–ethanol–water solvent, is primarily 18:1 with respect to both the alk-1-enyl moiety and acyl moiety (see Discussion) and that this composition accounts for its differential rate of staining with the plasmalogen reagents. The corresponding lyso compounds of PE-1 and PE-2, appear to be resolvable but to a less satisfying degree than are the parent substances.

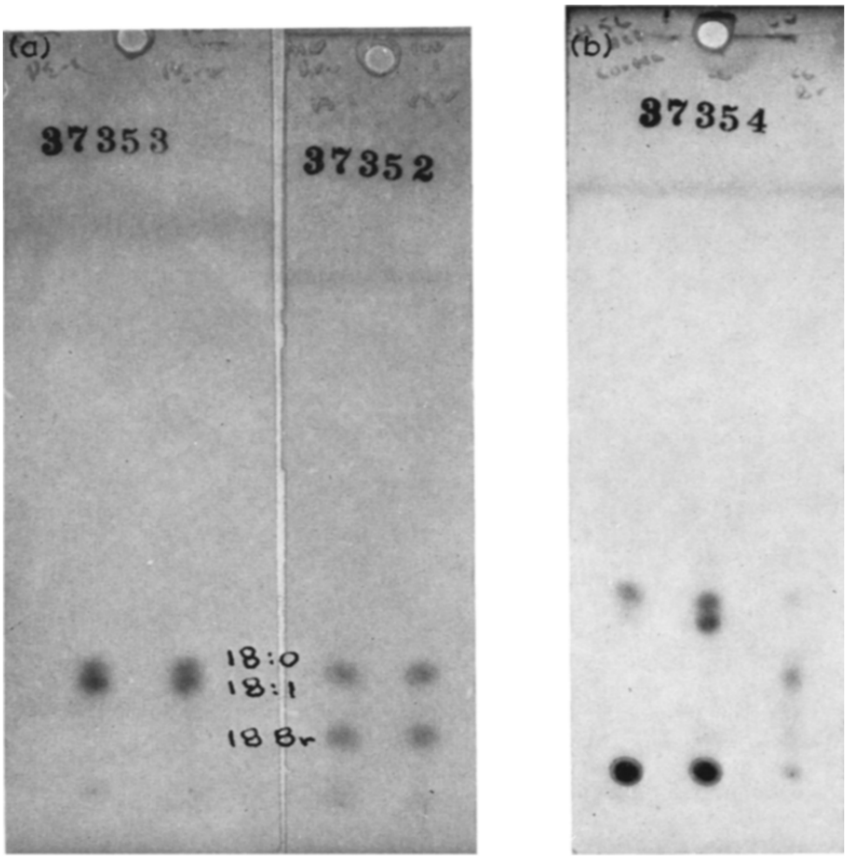

Fig. 5. Schleicher & Schuell F1500 chromatograms to demonstrate the aldehydes produced by the *in situ* HCl hydrolysis of (a) the PE-1 and PE-2 isolates of Fig. 4 and (b) from left to right, human cerebral cortex, corpus callosum. Chromatographic development was in isooctane–isopropyl acetate (100:1) and staining of the free aldehydes with the Schiff reagent; (b) had then been exposed to OsO_4 vapors where the polyunsaturated fatty acid of the cortex PE is shown at the origin. The reduced R_F value of the brominated 18:1 is obvious.

DISCUSSION

There have been some rather extensive gas chromatographic analyses of the acyl and alk-1-enyl content of the PE from human brain grey and white matter[10,11], bovine dorsal roots[12], whole bovine and mouse brain[13,14] and an analysis of the 18:1 aldehydes obtained from human brain and heart PE[15]. Of special interest is the observation[15] that the 18:1 alk-1-enyl of human PE was dominantly Δ-11,12 (*i.e.*, ω-7 series) whereas that of human heart consisted largely of the ω-9 series Δ-9,10. We interpret these data as providing supporting evidence for the presumed composition of PE-2 as described here and to imply that the PE plasmalogen of human brain (and presumably of myelin alone, from whatever source) has special physiological characteristics.

It would now be important to verify precisely that the PE-2 plasmalogen is entirely 18:1 for both radyl groups and, further, to determine the positions of the

unsaturation. The demonstration of clearly resolvable molecular species of PE plasmalogen, correlated with the myelin sheath, should re-focus the attention of investigations on the myelination process and on the demyelinating diseases to include this substance.

It has already been shown, for example, for the myelin-deficient Jimpy[16] and Quaking[17,18] mice that there are diminutions in PE plasmalogen and for Quaking[19] mouse a diminution in 18:1 fatty acid. Kishimoto *et al.*[20] have demonstrated, in the developing rat, an increasing prominence of brain phosphatide 18:1 fatty acid. How much of this data can be related to the PE-2 plasmalogen content remains to be established.

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