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Note

Application of methylamine O-deacylation and Vitride reduction to the analysis of neutral lipids: a thin-layer chromatographic evaluation

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Clarke and Dawson [1] have shown methylamine to be useful in the study of phospholipids in extracts of animal tissues. This requires the retrieval of the water-soluble phosphorus-containing hydrolysis products for ionophoretic analysis, basically resembling an earlier alkali metal hydroxide alcoholysis chromatographic methodology [2].

This recognition that methylamine is a practical phospholipid hydrolyzing reagent, limited to O-deacylation and not requiring subsequent neutralization, led us to explore more fully its application to other lipid groups as determined by thin-layer chromatography (TLC) of the water-insoluble (lipid) products. First, we empirically determined the conditions under which diacylphospholipids could be completely deacylated and 1-alkenyl-2-acyl-3-glycerophospholipids (plasmalogens) quantitatively converted to their respective lyso forms. We then selected a number of tissues notable for their diverse neutral lipid composition to serve as test substances for the method; the results are reported here. By comparing these observations with those obtained by Vitride reduction [3], we have a TLC procedure of value in deducing the structure of these respective neutral lipids which consist largely of various combinations of O-acyl, O-alkyl and glycosidic bonds with glycerol or other non-ionic hydroxy compounds. The effect of methylamine on plasmalogens, for example, is to mimic phospholipase A_2 while that of Vitride is to mimic the combined sequential effects of phospholipase C and lipase with the released fatty acids being converted into N-methyl fatty acid amides and reduction to long-chain alcohols, respectively (Fig. 1).

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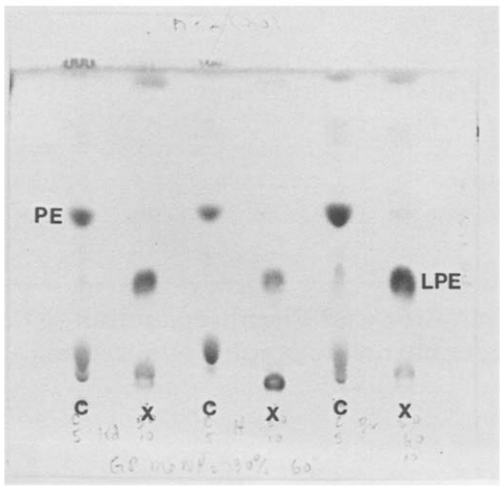

Fig. 1. Chromatogram developed in ethyl acetate–2-propanol–chloroform–methanol–water (5:5:3:2:1.8) of control (c) and methylamine-processed (x) extracts of guinea pig tissue. From left to right, as c/x pairs, kidney, heart, brain. Visualization was by the mercury chloride–Schiff plasmalogen procedure with Biebrich Scarlet counter-staining, showing, mainly, the conversion of phosphatidylethanolamine (PE) plasmalogen to lyso PE plasmalogen by the methylamine deacylation.

EXPERIMENTAL

A number of tissues of known complex neutral lipid composition [4–8] from mouse, rat, guinea pig and rabbit were selected to test the deacylation efficiency and specificity of methylamine to yield polar compounds applicable to TLC analysis. The skin and hair samples were extracted directly with acetone and the mouse preputial gland was extracted directly with chloroform–methanol (2:1). The remaining tissues and fluids were freeze-dried prior to extraction with chloroform–methanol as described earlier [9,10]. The TLC procedure, using 10 cm × 10 cm Macherey-Nagel (Duren, F.R.G.) Polygram Sil G plastic-backed sheets, has been described [9] as have the periodic acid Schiff (PAS) spot-tests for 1,2-glycols and the mercury chloride–Schiff reaction for plasmalogens (1-alkenyl) [10]. Desaga (Heidelberg, F.R.G.) developing tanks for 10 cm × 10 cm plates were used unlined and without prior equilibration. Mobile phases consisted of (a) hexane–diethyl ether (30:5), (b) chloroform–acetone (2:1) and (c) ethyl acetate–2-propanol–chloroform–methanol–water (5:5:3:2:1.8) as indicated in the figure legends.

The methylamine procedure, as follows, required 13 mm × 100 mm screw-capped tubes (PTFE seal) as the reaction vessel. The solvent from an appropriate aliquot (e.g., 1 ml) of extract was removed at 40 °C with a gentle stream of nitrogen. The lipid was then redissolved in 0.5 ml of 1-butanol, to which was added 1 ml of 40% methylamine in water (Aldrich, Milwaukee, WI, U.S.A.), and reacted for 60 min at 50 °C with frequent vortex-mixing. A 0.5-ml volume of benzene (or isooctane) was then added followed by 4.5 ml of 0.85% aqueous sodium chloride saturated with 1-butanol. Following vigorous shaking the butanol–benzene (or butanol–isooctane) containing the lipid separated as the top layer and was di-

rectly sampled, e.g. 10 μl, for TLC examination by development in chloroform–acetone (2:1) and stained by the PAS reaction. Methylamine has a boiling point of $-6°C$ and is consequently not retained by the chromatogram after application of the sample. The Vitride procedure has been described elsewhere [3] and resembles in a general way the methylamine procedure just described. Vitride is sodium bis(2-methoxy)aluminum hydride. It is available as 3.4 *M* in toluene (Aldrich). This concentration is too viscous for volumetric use so must be freshly diluted with benzene to 0.34 *M*.

Lipid standards

Alkane diols were prepared from 2-hydroxypalmitic acid, 12-hydroxystearic acid and 12-hydroxyoleic acid by Vitride reduction. N-Methyl fatty acid amides were prepared by the above described alkaline O→N-transacylation procedure from various fatty acid methyl esters. These starting materials were obtained from Serdary Research Labs. (London, Canada), as were the standards used to generically characterize zones 1–4 of Fig. 4 and some portions of the chloroform–acetone (2:1) TLC spectrum of Fig. 6.

RESULTS

Preparative TLC, using hexane–diethyl ether (30:5) development of the white and pink portions of rabbit harderian gland, provided Vitride and methylamine

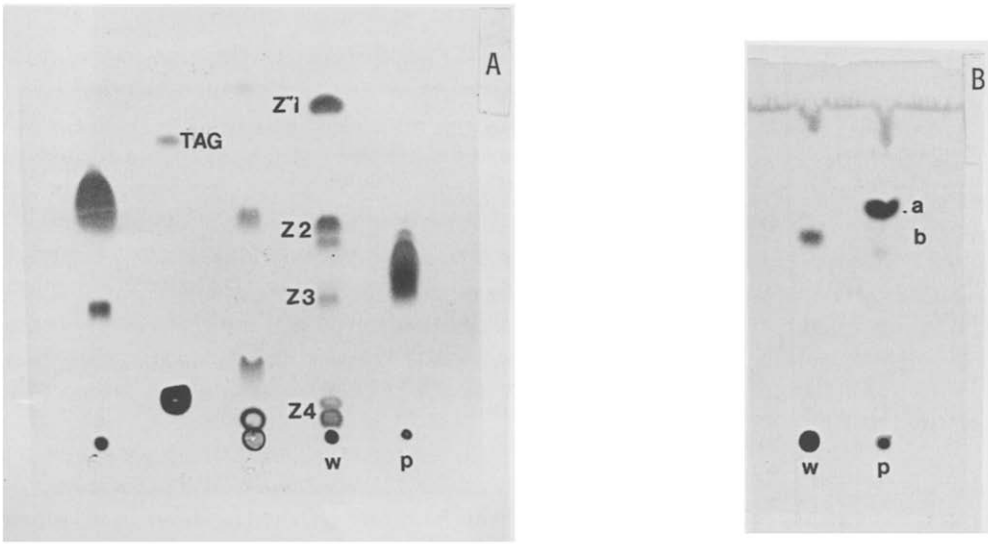

Fig. 2. (A) Chromatogram developed in hexane–diethyl ether (30:5) with vapor-phase osmium tetroxide–Biebrich Scarlet visualization. From left to right, guinea pig whole harderian gland, TAG and oleyl alcohol standards, guinea pig hair, white (w) and pink (p) portions of rabbit harderian gland. The zonation (Z1–4) referred to in the text is indicated. (B) w and p as above, developed in chloroform–acetone (2:1) and visualized by the PAS reaction to show the free 1-(O-acyl)-HAG (a), perhaps representing an in vivo esterolytic limitation, and MAG (b).

data to indicate that the Fig. 4 zone 1 contained 2-hydroxy fatty acid wax esters, zone 2 contained both 2-hydroxy fatty acid and 1-alkyl glycerol (MAG) compounds, zone 3 consisted of triacyl glycerol (TEG) and the zone 4 lipids yielded

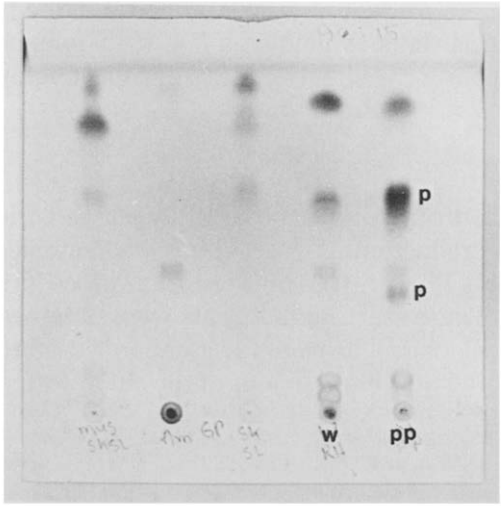

Fig. 3. Same TLC conditions as Fig. 2 but with mercury chloride-Schiff-Biebrich Scarlet visualization. The respective zones are recognizable from the w rabbit harderian lane. From left to right, mouse skin surface lipids, guinea pig amnion, guinea pig skin surface lipids, white (w) portion rabbit harderian gland and male mouse preputial gland (pp) where only zones 2 and 3 of pp have alkenyl (p) components.

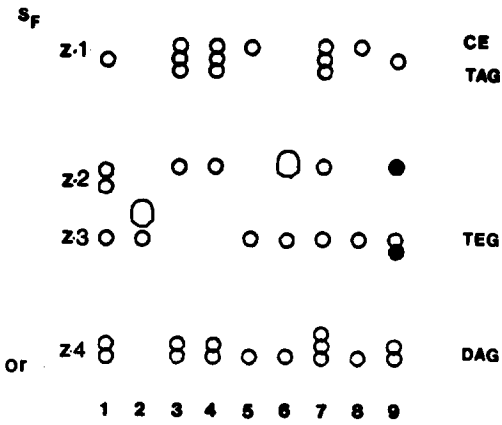

Fig. 4. Summarizing chart of TLC in hexane–diethyl ether (30:5). Or and S_F are origin and solvent front, respectively, zones 1–4 are indicated at the left and standard positions are indicated at the right where CE is cholesterol oleate, TAG is trialkyl glycerol, TEG is triacyl glycerol, and DAG is 1,2-dialkyl glycerol. Lanes: 1 = white portion rabbit harderian gland; 2 = pink portion rabbit harderian gland; 3 = mouse skin surface lipids; 4 = guinea pig skin surface lipids; 5 = guinea pig amnion; 6 = guinea pig harderian gland; 7 = guinea pig fetal skin surface lipids; 8 = mouse skin surface lipids; 9 = male mouse preputial gland. The closed circles represent plasmalogen (alkenyl)-containing components.

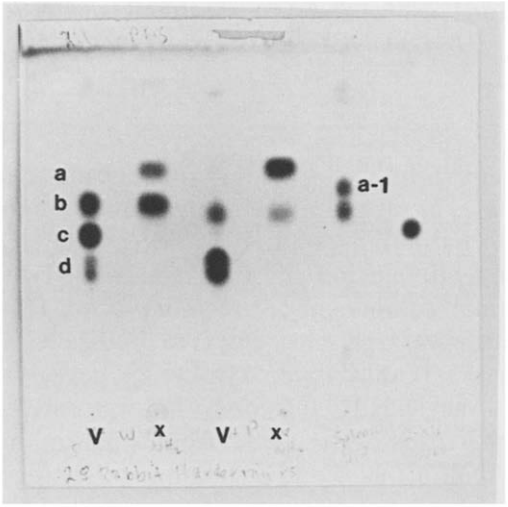

Fig. 5. TLC using chloroform–acetone (2:1) as mobile phase and PAS for detection. From left to right, white portion Vitride (V) and methylamine (x) pair and pink portion (V/x pair) rabbit harderian gland of which (a) is 1-(O-acyl)-HAG, (b) is MAG, (c) is 1,2-alkane diol, (d) is HAG. Lane 5 is the Vitride pattern from guinea pig spleen where a-1 is 1-alkenyl glycerol derived from plasmalogen and the 6th lane is 1,2-alkane diol (16:0).

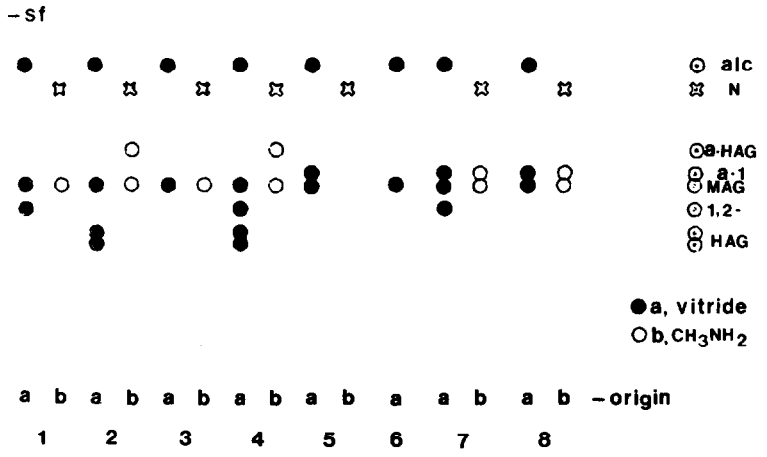

Fig. 6. Summarizing chart of Vitride and methylamine products from the following specimens: (1) white and (2) pink portion of rabbit harderian gland, (3) guinea pig harderian gland (same pattern for guinea pig hair, fetal skin surface lipids and amniotic fluid), (4) adult rabbit hair, (5) rabbit fetal skin surface lipids, (6) human amniotic fluid, (7) human amnion, (8) male mouse preputial gland. The spot identity is indicated at the right where alc is fatty alcohol, N is N-methyl fatty acid amide, a-HAG is 1-(O-acyl)-HAG, a-1 is 1-alkenyl glycerol, MAG is monoalkyl glycerol, 1,2 is 1,2-alkane diol (16:0) and HAG is hydroxyalkyl glycerol. The zonal (Fig. 4) origin of each of these components has not been completely established.

mostly 2-hydroxy fatty acids and hydroxyalkyl glycerol (HAG). The interzone (2–3) material of the pink portion had HAG as the Vitride product and 1-(O-acyl)-HAG as the methylamine product (cf. Fig. 6), as the respective derivatives

from the initial triacyl-HAG. The legends of Figs. 2–6 provide details of the remaining observations.

DISCUSSION

Our results are essentially in agreement with the observations of Kasama et al. [5], Rock and Snyder [6] and Rock et al. [7] regarding the neutral lipid content of the pink and white portions of rabbit harderian gland (Figs. 2B, 5 and 6); in addition, we observed free MAG in the white portion and free 1-(O-acyl)-HAG as a major component and MAG as a small component of the pink portion (Fig. 2B). This is presumably reflecting some as yet undisclosed in vivo process relating to the synthesis and/or catabolism of 1-(O-acyl)hydroxyalkyl-2,3-acyl glycerol/1-alkyl-2,3-acyl glycerol. The 1-(O-acyl)-HAG interpretation was derived from its R_F and PAS characteristics. The Vitride-derived HAG from female rabbit harderian gland was resolved into two spots (Figs. 5 and 6) possibly reflecting a more favorable ratio (5) of the 16:0/18:0 alkyl components than occurs in the male. These two portions of the rabbit harderian gland are clearly morphologically separable and are shown to be differentiable on the basis of their neutral lipid content so that cross-contamination can be readily detected (Fig. 5).

Nikkari [4] has reported hair lipids of rabbit (a) to contain hydroxy fatty acid diesters and both mouse (b) and guinea pig (c) to contain diester alkane diols. Our Vitride and methylamine observations confirm a and b but not c. The longer chain length of the mouse diols [4] presumably increased the R_F bringing these diols into the MAG region. Co-chromatography with 16:0 diol and/or MAG were helpful in resolving this issue. Fig. 6 tabulates our additional observations with rabbit hair as containing lipids characterizing whole rabbit harderian gland, as indeed its hexane–diethyl ether TLC pattern indicated.

The guinea pig surface lipids have been reported [8] to contain both alkane diol wax esters and glycerol ether diesters; our specimens (Fig. 6) show only MAG to be present. The data on guinea pig harderian gland are reported here for the first time.

A number of analytical procedures, in addition to TLC, were employed by others, as just described. Our confirmation of some of the results reported and failure to confirm others suggests that a closer look at all of the methodology, as applied to these substances, is required.

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