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Note

Analytical separation of green-plant and animal neutral lipids by thin-layer chromatography

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Green plant tissue contains a complex mixture of polar and neutral lipids[1]. Although the polar lipids consist of fairly characteristic glyco- and phosphoglycerolipids[2], the neutral lipids are a heterogeneous mixture in which chlorophyll and carotenoid prenylpigments predominate over far smaller amounts of non-colored neutral lipid, notably sterol[3]. Co-migration of both pigmented and non-pigmented neutral lipids in solvents designed to resolve the polar lipids makes separation of plant neutral lipids a particular problem. Varying degrees of plant neutral lipid resolution can be attained by multiple rounds of thin-layer[4] or column[5] chromatography or by countercurrent distribution[6], but the multi-step nature of these procedures makes them laborious at best, invites degradation of the lipids themselves and jeopardizes quantitative recoveries.

During our studies on the lipids of the green phytoflagellate *Chlamydomonas reinhardtii*, we devised a thin-layer chromatographic (TLC) system which separates out of the total green-plant tissue lipid extract the neutral lipids from the polar glycerolipids and simultaneously resolves the carotenoids and chlorophylls into discrete bands[7]. Since polar lipid remains at or very near the origin while the resolved pigments are free from non-colored lipid contamination, the question arose as to the behavior of non-pigmented neutral lipid in this TLC system. We detail this behavior here and demonstrate that resolution of the major non-pigmented neutral lipid classes from both polar glycerolipid and green-plant chlorophyll and carotenoid pigment can be attained. Furthermore, resolution of the neutral lipid classes commonly encountered in animal tissues free from polar lipid can also be readily achieved in one step out of a total-tissue lipid extract.

EXPERIMENTAL

Tissues and lipid extraction

The chlorophyte *Chlamydomonas reinhardtii* 137^+ was grown in axenic, photosynthetic culture as described[7]. Fed (*i.e.*, not fasted prior to sacrifice) male rats from the Yale Medical School colony were the source of liver.

Total tissue lipids were extracted and purified from pelleted algae and from

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fresh liver homogenate prepared according to Jelsema and Morré[8] by a modified Bligh–Dyer procedure[9]. The final chloroform phases represent >98% recovery of tissue lipid. Purified lipid was stored in darkness below −20°C and sealed under nitrogen for no more than 24 h prior to chromatography.

Lipid chromatography

Total neutral lipid and total polar lipid were separated out of the liver lipid extract by one-dimensional TLC with the solvent of Marinetti[10].

The TLC system studied here has been described previously by us[7]. In brief, chromatography was carried out on glass TLC plates precoated with an 0.25-mm layer of Merck Type-60 F-254 silica gel containing fluorescent indicator (E. Merck, Darmstadt, G.F.R.). The components of the developing solvent, benzene–isopropanol–water (100:10:0.25, v/v/v), were of analytical grade as purchased. Lipids (concentrated in acetone or chloroform) were applied manually to the plate as a streak containing ⩽250 μg total lipid. Development was carried out (in darkness for the plant lipids) at room temperature in an equilibrated tank until the solvent migrated to the opposite edge of the plate (20 cm, ≈1 h). When necessary, lipids were quantitatively eluted by extracting the gel[9].

Lipid detection, identification and quantitation

Lipids were visualized non-destructively under ultraviolet (UV) light or with iodine vapor[11] and destructively by acid–bichromate charring[12].

Pigments were identified by their absorption spectra as detailed[7]. Non-pigmented lipids were identified by several criteria: chromatographic behavior with respect to standards; specific dyeing reagents for phosphorus[13] and sterol[14]; analysis of glycerolipid deacylation products by paper chromatography[15], TLC[16] and dyeing reagents; and chemical determination of ester group: glycerol ratio.

Glycerolipids and sterol ester were quantitated by hydroxamate ester assay[17], free fatty acid by a Rhodamine procedure[18] and sterol by the Liebermann–Burchard reaction[19]. Lipid phosphorus[20] and glycerol[21] were determined by colorimetric microassay. Radioactive lipids were quantitated by liquid scintillation spectrometry using Bioflour (New England Nuclear, Boston, MA, U.S.A.) as scintillant[7].

Lipid saponification

Mild saponification of lipid was accomplished with alcoholic base[5].

Lipid standards

Lipids of the highest grade were purchased in non-radioactive form from Serdary Labs. (Ontario, Canada), Nu-Chek Prep. (Elysian, MN, U.S.A.) and Sigma (St. Louis, MO, U.S.A.). All radioactive lipids were from New England Nuclear and were verified >98% radiochemically pure in the appropriate solvents specified by the manufacturer.

RESULTS AND DISCUSSION

The ability of our TLC system to separate the major classes of neutral lipid was demonstrated by chromatographing non-labeled and isotope-radiolabeled commercial

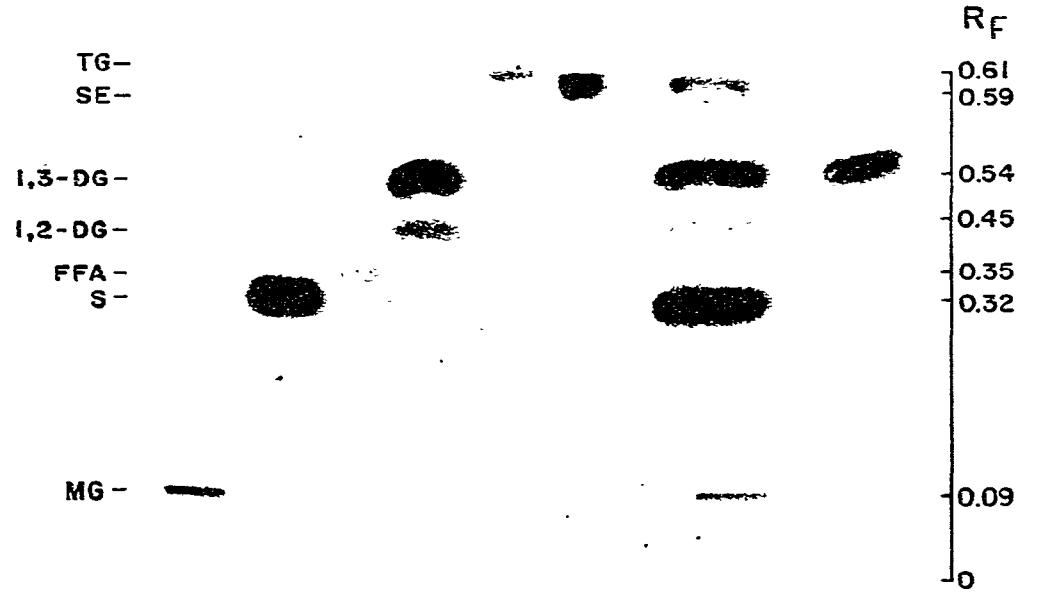

Fig. 1. One-dimensional separation of neutral lipid standards in the solvent benzene–isopropanol–water (100:10:0.25, v/v/v) on a 20 × 20 cm glass TLC plate precoated with Merck Type-60 F-254 silica gel. Abbreviations: MG = monoglyceride; S = sterol; FFA = free fatty acid; 1,2-DG = 1,2-diglyceride; 1,3-DG = 1,3-diglyceride; SE = sterol ester; TG = triglyceride. The R_F value corresponding to each lipid is also given. Detection: acid–bichromate charring.

standards (Fig. 1). The four neutral glycerolipids (monoglyceride, 1,2-diglyceride, 1,3-diglyceride and triglyceride) are resolved along with sterol, sterol ester and free fatty acid. Recovery of standards after chromatography was complete as determined both by appropriate biochemical assay (for ester group, glycerol, sterol and fatty acid) and by quantitation of the radioactivity applied to the chromatogram and associated with the resolved, labeled lipid standards. Over a wide range ($\approx$5–250 μg total lipid), discrete bands are obtained for each neutral lipid class applied either individually or as various mixtures. No trailing, smearing or shadowing is ever observed, and no neutral lipid remains at the origin, even when the developing chamber is not saturated with solvent. The quantitative recoveries and observed chromatographic behavior demonstrate retention of neutral lipid integrity through the TLC procedure.

While neutral lipids move readily and completely from the origin (Fig. 1), polar lipids (phospholipid, glycolipid) are fully retained at or very near the origin ($R_F \leqslant 0.035$, Fig. 2a), as previously demonstrated[7]. Our interests in the biogenesis of *Chlamydomonas* thylakoid membrane[22] and hepatocyte endoplasmic reticulum[23] prompted us to apply this TLC procedure to green-plant and liver tissues with the purpose of resolving the neutral lipids out of the respective total-tissue lipid extracts (Fig. 2). As we have reported[7], this TLC system resolves each pigmented neutral lipid ($R_F \geqslant 0.07$) from polar lipid ($R_F \leqslant 0.035$) out of the total cellular lipid extract of *Chla-*

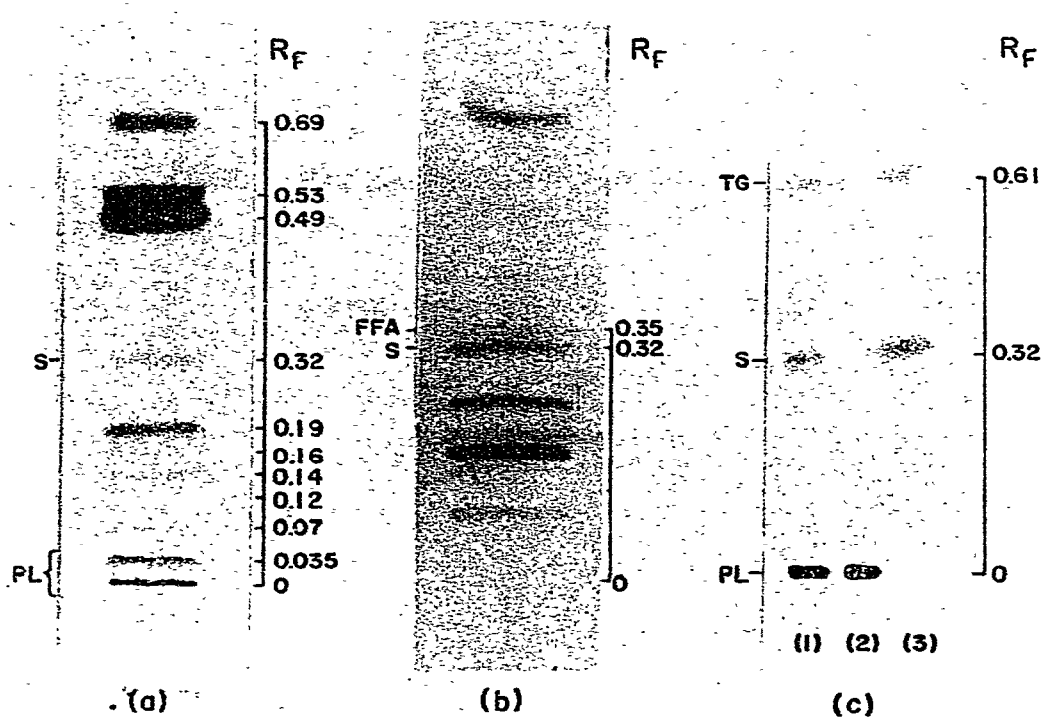

Fig. 2. One-dimensional chromatography of green-plant and animal tissue lipids. Chromatographic parameters are as in Fig. 1. a, Whole-cell lipid extract of the green alga *Chlamydomonas*. Unidentified bands (R_F = 0.07–0.19, 0.49–0.69) are chlorophyll and carotenoid pigments and have been identified elsewhere[7]. b, The organic phase recovered from mild base saponification of the *Chlamydomonas* whole-cell lipid extract. Unidentified bands (R_F = 0.07–0.19 and 0.69) are the unsaponified carotenoids. c, Rat liver lipids; lanes: 1, rat-liver total lipid extract; 2, total rat-liver polar lipid; 3, total rat-liver neutral lipid. The total polar and total neutral lipid fractions were prepared from the rat-liver total lipid extract by an independent TLC method[10]. Abbreviations: PL = polar lipid; S = sterol; TG = triglyceride; FFA = free fatty acid. Detection: acid-bichromate charring.

mydomonas (Fig. 2a). The chromatogram in Fig. 2a further illustrates that the virtually exclusive[24] neutral lipid class of the alga, sterol, can be well-resolved at R_F = 0.32 out of the total green-plant lipid extract, free of the neutral prenylpigments which migrate from the origin zone and free of the polar lipids which do not. The sterol band co-migrates with authentic sterol, gives a typically positive Liebermann–Burchard reaction and is negative to glycerol assay. The amount of sterol in the total-cell lipid extract applied is fully recovered in the band; thus, quantitative sterol resolution is obtained. Re-chromatography of the eluted sterol band alone in this system and in others[25] always yields a single band.

Saponification of the *Chlamydomonas* cellular lipid extract removes the polar glycerolipids and chlorophylls, leaves the carotenoids and sterols unreacted and releases fatty acid from acyl lipid. Chromatography of the total organic phase after mild base saponification of *Chlamydomonas* cellular lipid (Fig. 2b) confirms the sterol separation obtained out of the total-cell lipid extract and illustrated in Fig. 2a. Additionally, the free fatty acid products of acyl lipid saponification are also resolved; as

determined by chemical assay, the total free fatty acid in the post-saponification organic phase and applied to the plate is fully recovered in the band at $R_F = 0.35$. The fatty acid band is negative for phosphate, sterol, glycerol and esterified fatty acid. Both the free fatty acids and the sterol are distinct from the non-saponifiable carotenoids ($R_F = 0.07$–0.19 and 0.69) simultaneously resolved[7].

Chromatogram c of Fig. 2 illustrates another application of the TLC method, resolution of the neutral lipids out of a total rat-liver lipid extract. Since the animals were not fasted prior to sacrifice, the neutral lipids consist virtually exclusively of triglyceride and sterol (*i.e.*, cholesterol)[26] and are present along with a polar lipid complement which is largely phosphatide[27]. The triglyceride and sterol are well-resolved both in the presence (lane 1) and absence (lane 3) of polar lipid; the resolved spots represent the total amounts of these neutral lipid classes in the applied tissue extract, as quantitated by the appropriate microassay. The liver's total polar lipid prepared by an independent TLC method[10] remains completely at the origin in a spot which contains all the phosphate in the lipid applied (lane 2).

From the results presented, we conclude that our combination of solvents and commercial TLC plate readily resolves the major non-pigmented neutral lipid classes out of total lipid extracts of both green-plant and animal tissue. The resolution attained is attributable to two properties of this TLC system: (a) only neutral lipid, and not polar lipid, migrates with the solvent during development; and (b) upon development, the migrating neutral lipid classes, unaltered biochemically, are quantitatively separated from one another. These characteristics ensure that polar lipid does not interfere with neutral lipid resolution (Fig. 2). Furthermore, the non-pigmented neutral lipids band at positions distinct from the chlorophylls and major carotenoids of green-plant tissue, as comparison of the R_F values for the non-pigmented neutral lipids (Fig. 1) and for the pigments (Fig. 2a) readily demonstrates.

While the variety of complex non-pigmented neutral lipids found in the plant kingdom (*cf.*, ref. 28) does not allow prediction that this TLC system alone will suffice every need for neutral lipid separation, the ease, rapidity and resolution of the one-step method would appear to give it wide significance for the analytical and preparative isolation of the major neutral lipid classes of green-plant and animal tissues. Since lipid integrity is retained during chromatography, the method readily serves as an efficacious initial step to subsequent investigation at the molecular level of the components of the resolved neutral lipid classes.

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