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Phospholipid analysis on a micro scale

Usually, phospholipids are separated by two-dimensional thin-layer chromatography on 20 × 20 cm plates[1-4] and after digestion the phospholipid phosphorus is quantitatively determined with ammonium molybdate (modifications of the method of FISKE AND SUBBAROW[5] and BARTLETT[6]).

Working with small amounts of membraneous material from various sources, it appeared desirable to develop a system which permits the analysis of phospholipids in the nanomole range. This was achieved by the use of two-dimensional thin-layer chromatography on 6 × 6 cm plates with an adsorbent of Silica Gel G plus calcium sulfate, followed by phospholipid phosphorus determination in a sample volume of 0.2 ml as described in this communication.

Experimental

The material was extracted according to FOLCH *et al.*[7]. The sample was then applied to 6 × 6 cm plates using a micropipet. The application point should not exceed 1.5 mm in height and 3 mm in width. The plates with the adsorbent composed of Silica Gel G (Merck, Darmstadt, G.F.R.) plus calcium sulfate (3:1) were activated at 120° for 20 min before use. As solvent systems we selected chloroform–methanol–acetic acid–water (30:10:0.4:1) or chloroform–methanol–water (30:10:0.5) in the first dimension and chloroform–methanol–28 % aq. ammonia (15:5:1) in the second. Development distance was about 5 cm in each dimension with 15 min air-drying in between. The spots were made visible under UV light by spraying with 0.05 % Rhodamine B in ethanol supplemented with 5 % Tinopal (Geigy AG, Frankfurt, G.F.R.) according to POPOV AND STEFANOV[8]. This reagent was found to be very sensitive for phospholipids[9]. The individual phospholipids then were carefully scraped from the plates and digested together with the adsorbent in small calibrated vials (I.D. 4 mm) in an electrically heated stove (Thermoblock with small bore holes, WTW, Weilheim, G.F.R.). Digestion and color development were performed essentially according to GERLACH AND DEUTICKE[10] with 0.06 or 0.03 ml of digestion reagent, 0.3 or 0.15 ml of 1 % ammonium molybdate solution, and 0.02 or 0.01 ml of reduction reagent with an exact final volume of 0.4 or 0.2 ml (distilled water added when necessary) for the main or minor components of the sample, respectively. The optical density was determined in micro cuvettes at 650 or 820 nm.

Nuclear membranes of pig liver were prepared as described elsewhere[11]. HeLa cells were grown in Roux bottles with Eagle's essential medium supplemented with 2 % fetal calf serum. Suspension cultures of the blue-green alga *Plectonema boryanum* were a gift from R. M. BROWN, Jr., Chapel Hill, N.C., U.S.A. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, lysophosphatidylcholine, sphingomyelin, and cardiolipin were purchased from Applied Science Lab. Inc., State College, Pa., U.S.A. Monogalactosyl diglyceride and digalactosyl diglyceride were gifts from G. UNSER, Freiburg, G.F.R.

Results and discussion

In Fig. 1 four representative two-dimensional chromatograms are presented

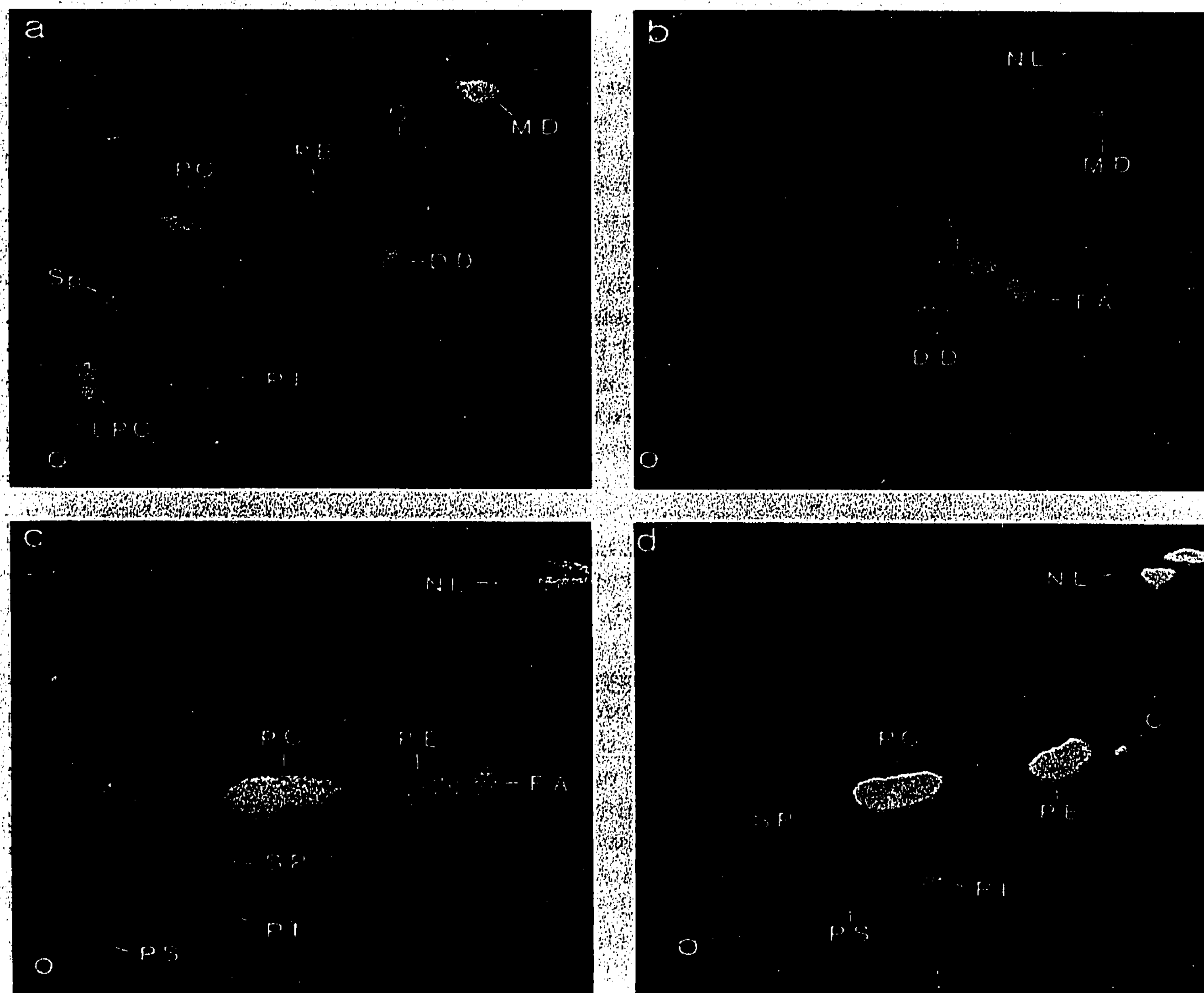

Fig. 1. Thin-layer chromatograms photographed under UV light. Original size. (a) Reference substances; (b) lipids of the blue-green alga *Plectonema boryanum*; (c) lipids of pig-liver nuclear membranes; (d) lipids of HeLa cells. Identification of components: PS = phosphatidylserine; LPC = lysophosphatidylcholine; PI = phosphatidylinositol; SP = sphingomyelin; PC = phosphatidylcholine; PE = phosphatidylethanolamine; C = cardiolipin; FA = fatty acids; DD = digalactosyl diglyceride; MD = monogalactosyl diglyceride; NL = neutral lipids; X = unknown; O = origin.

which demonstrate the usefulness of the method described. As generally can be observed, variations in temperature and humidity may cause small shifts of the spots. The separation and the sharpness of the spots is greatly enhanced by mixing Silica Gel G with calcium sulfate, compared with pure Silica Gel G layers. With the highly sensitive Rhodamine B–Tinopal spray less than 0.1 nmole phospholipid can be detected. This spray, however, cannot be used with the solvent mixtures of PARSONS AND PATTON[4] which also give good separations in our system, since the sensitivity of this spray is diminished under acidic conditions.

In Figs. 1a and 1b the plastidal galactolipids monogalactosyl diglyceride and digalactosyl diglyceride are also chromatographed together with some phospholipids.

These glycolipids can be quantitatively estimated by the method of ROUGHAN AND BATT[22].

The phospholipid composition of HeLa cells determined by a macro method and by the micro method described in this communication is listed in Table I. As can be seen, the results of both methods agree well, and the standard deviations are only slightly increased with the micro method.

TABLE I

PHOSPHOLIPIDS OF HeLa CELLS: COMPARISON OF THE RESULTS OF QUANTITATIVE ANALYSES AFTER THE MICRO METHOD AND A MACRO METHOD (% OF TOTAL PHOSPHOLIPID)

Phospholipid	*Micro method*[a]	*Macro method*[a]
Phosphatidylcholine	52.1 ± 1.9	51.0 ± 1.5
Phosphatidylethanolamine	23.5 ± 1.7	24.8 ± 1.6
Sphingomyelin	9.1 ± 2.4	8.7 ± 1.9
Phosphatidylinositol	7.0 ± 1.4	6.3 ± 1.3
Phosphatidylserine	5.7 ± 2.1	4.2 ± 1.4
Cardiolipin	3.3 ± 1.6	3.7 ± 1.3

[a] Mean values ± S.D. of six analyses.

With our method less than 0.1 nmole phospholipid can be visualized on the thin-layer plates, and 0.5 nmole phospholipid can be measured quantitatively. Depending on the different content of individual phospholipids in biological material 30–50 nmole phospholipid are required for one analysis in order to also determine the minor compounds.

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