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GENERAL METHOD FOR THE PURIFICATION OF LIPIDS FOR SURFACE PRESSURE STUDIES

APPLICATION TO MONOGALACTOSYLDIGLYCERIDE

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SUMMARY

In order to prepare lipids free of surface-active contaminants and suitable for the measurement of surface pressure isotherms a general purification method has been developed involving preparative thin-layer chromatography. A detailed description of the procedure and blank experiments carried out with a monolayer trough are presented. The method provides a rapid purification of various classes of lipids, the whole procedure taking less than 1 day. It gives highly reproducible results, better than 2 Å^2 per molecule at any surface pressure. As an example of an application, the results obtained with a commercial sample of monogalactosyldiglyceride, a major plant lipid, are reported.

INTRODUCTION

In any work concerned with monolayer properties of pure compounds or mixtures of surface-active components, clearly the purity of the lipid products is important and blank experiments should be performed, as emphasized by Gaines[1]. The contamination of lipids by extraneous surface-active agents collected or concentrated in the many steps between the extraction of the lipids and their monolayer study is certainly the most important problem. When one deals with a monolayer film, containing about 10^{16} molecules of material, a small trace of surface-active contaminants can cause serious errors. Therefore, substances adsorbed on the glassware used in the extraction and purification procedure or present in the adsorbents (silica gel, alumina, etc.) or in the various solvents used are all likely to be found as contaminants in the "purified" material at the end. Therefore, much care must be taken and blank experiments must be performed on a monolayer trough at each step of the purification process to reveal the presence of impurities. Consequently, the purification process should be limited to the minimum number of steps in order to avoid the above problems.

This paper deals with a general method for the purification of lipids with the

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aim of performing surface pressure studies. The technique uses preparative thin-layer chromatography (TLC). It can either be used to purify lipid samples available from commercial suppliers or used in the last step of an extraction procedure, when ultrapure lipid products free from surface-active contaminants suitable for monolayer studies are required. The overall purification method can be performed within 1 day, the surface pressure isotherms obtained being highly reproducible, thus readily allowing the use of samples coming from different purification batches. A detailed description of the procedure together with the basic blank experiments involved are presented. The technique was applied to the purification of a commercial sample of monogalactosyldiglyceride (MGDG), a major lipid of the thylakoid membrane. The results and efficiency of purification are compared with literature values. A column chromatographic purification technique, a more lengthy process, is also described and the results of the two techniques are compared.

MATERIALS AND METHODS

The Langmuir trough

The surface pressure isotherms were measured on a float-type torsion balance. An aluminium trough (Cenco Co. Montreal, Canada, 61.9 × 13.2 × 1.5 cm) coated with PTFE (Commercial Plastic and Supply Co., Montreal, Canada) and mounted on a vibrationless granite table was used. The table was placed on low-profile pneumatic spring mounts (Barry Wright Corp. Montreal, Canada, Model SLM-1). The surface pressure was detected through a Mylar float attached to the walls of the trough with PTFE sheets (0.075 in., Carborundum Co., Farmingdale, NY, U.S.A.). The float was mechanically coupled to a stainless-steel torsion wire (0.056 cm diameter). A linear magnetic transducer (Hewlett-Packard, Pointe-Claire, Canada, Model 7V DCDT) converted the torsion angle to a voltage detected by a Keithley Model 172 multimeter. The sensitivity of the detection system was 12.3 mN m^{-1} V^{-1}. The lipid was deposited on a phosphate buffer sub-phase (10^{-3} *M*, pH 7.8) prepared with quartz-distilled water (specific resistivity greater than 18×10^6 Ω cm; surface tension greater than 71 mN m^{-1}). The temperature was kept at 20.5 ± 0.5°C throughout the experiment.

Glassware cleaning procedure

All of the glassware used in the purification procedure was cleaned using either the usual sulphuric–chromic acid mixture or a solution of No-Chromix Compound (Godax Lab., New York, NY, U.S.A.) dissolved in concentrated sulphuric acid, followed by thorough rinsing (ten times with tap water) and soaking for about 15 min in distilled water. The glassware was dried in an oven at 100°C. Both techniques gave identical results. The efficiency of the cleaning process was checked by concentrating under nitrogen 50 ml of Aristar-grade diethyl ether (BDH, Montreal, Canada; tested for surface-active contaminants as described below) down to 1 ml in a cleaned erlenmeyer flask; 100 µl were then deposited at the air–water interface on the Langmuir trough and no contamination from the glassware was found.

Solvent tests

Analytical-reagent grade chloroform and methanol were bought from Canadian Labs. (Montreal, Canada). Chromatographic-grade benzene from Fisher Scientific

(Montreal, Canada) and Aristar diethyl ether from BDH were also used. All of the solvents were checked from the bottle for surface-active contaminants according to the following procedure: 50 ml of the solvent (*i.e.*, the volume of solvent from which, after evaporation, the purified lipid was obtained) were transferred into a cleaned erlenmeyer flask and evaporated to dryness under nitrogen. The residue was dissolved into 1 ml of Aristar diethyl ether and 100 μl were deposited at the air–water interface on the Langmuir trough. Except for the diethyl ether, which was found to be satisfactorily pure in this test (less than 0.5 mN m^{-1} for the moveable barrier 1.5 cm away from the float), all of the solvents gave a significant response (typically 6–8 mN m^{-1} 10 cm away from the float). Purification of the solvents was successfully achieved by distillation, using a 30-cm Vigreux column. The purity of the distilled solvents was then improved considerably (about 0.5 mN m^{-1}, 1.5 cm away from the float).

Silica gel purification

The silica gel G (40–140 mesh; Fisher Scientific) used for the column chromatography and the silica gel on the TLC plates (15 × 20 and 5 × 20 cm plates, 500-μm layer; Analtech, distributed by Mandel Scientific, Montreal, Canada) used in the preparative chromatography were found to have surface-active contaminants. The silica gel for column chromatography was treated according to a procedure described by Carreau *et al.*[2]. The silica gel (50 g) was treated for 30 min at 60–70°C with 200 ml of 0.2% sodium ethylate in purified ethanol (see above). The silica gel was filtered over a sintered-glass Büchner funnel (fine porosity) and treated under the same conditions with 200 ml of 20% acetic acid in purified ethanol. The final product was filtered and washed repeatedly with purified ethanol, then with distilled water and dried over a cleaned watch-glass at 110°C in an oven for about 12 h. The column was mounted in the elution solvent (chloroform–methanol–water, 90:25:2). The elution solvent (50 ml) was collected and tested for surface-active contaminants as described above. No significant difference was observed between the 50-ml fraction eluted from the column and the same amount of purified solvent tested in the same way, thereby showing the effectiveness of the purification.

The TLC plates used for the preparative chromatography of the lipid were washed by two successive developments using methanol–water (50:50) in a clean elution chamber. A blank experiment was performed with the TLC plates to ensure that this treatment was efficient in removing the adsorbed surface-active contaminants. A band of about 80 cm^2 of silica gel, corresponding to the amount normally used in an actual purification, was scraped off the plate. The possible contaminants were desorbed by stirring the silica gel in chloroform–methanol (65:35) for about 15 min. The silica gel was then filtered off on a clean sintered-glass funnel, the solvent evaporated to dryness and the residue dissolved in 1 ml of purified benzene. A 100-μl volume of this solution was deposited on the Langmuir trough and no significant contamination was found.

Purification methodology

Preparative chromatography. The plates used for the preparative TLC were cleaned as described above. MGDG (5 mg, from spinach leaves; Serdary Co., London, Canada) dissolved in 100 μl of benzene was deposited spotwise on two 500-μm plates (15 × 20 cm). About fifteen spots 7–8 mm apart were deposited on each plate, using

3 μl of the benzene solution per spot. Two other plates (5 × 20 cm) with one spot of the lipid solution on each were also used as a reference to locate the exact migration position of the lipid. These four plates were put in a migration tank lined with filter-paper. The chromatograms were developed with chloroform–methanol–water (90:25:2). The spots on the reference plates were then revealed using iodine vapour. The lipid on the chromatograms could therefore be located with precision. The commercial sample showed two narrowly separated spots, characteristic of the various aliphatic chains of MGDG[3] (R_F = 0.72 and 0.69), together with coloured pigments (probably carotenoids) in front of these spots and a few more spots of unidentified material, with lower R_F values. A band about 8–10 mm wide containing the lipid was scraped off the plate. The lipid was desorbed from the silica gel by stirring the gel for about 15 min in chloroform–methanol (65:25, v/v), the solution was filtered on a sintered-glass funnel and the solvent evaporated under nitrogen. Typical yields (amount of purified material/amount of starting material) ranged from 55 to 60%. The lipid, showing only the two close characteristic spots by TLC, was stored in solution in benzene under nitrogen at −20°C in the dark. It was always used within 10 days after purification as degradation was apparent by TLC when it was kept for a longer period.

Column chromatography. A 50-g amount of silica gel, purified as described above, was slurried by addition of chloroform–methanol–water (90:25:2) and poured into the chromatographic column (2.5 × 30 cm). A 50-mg amount of MGDG was dissolved in about 1 ml of chloroform and deposited carefully on top of the column. The lipid was eluted at a rate of about 1 drop per second using chloroform–methanol–water (90:25:2). Fractions of 3 ml were collected and analysed by TLC, the spots on the plates being revealed using iodine vapour. Care was taken to minimize contact of the lipid with air and each fraction was flushed with nitrogen. The test-tubes were kept sealed until they were analysed by TLC for their lipid content. The fractions kept showed spots characteristic of MGDG, together with a very faint spot also observed where the sample was deposited on the plate. The origin of this spot and its consequences for the monolayer properties of MGDG are discussed below. Typically, the purified lipid was found in about 5–7 fractions. These fractions were collected together and the solvent was evaporated under nitrogen. The overall yield of the purification was about 50%. The lipid was kept in purified benzene as described above.

Preparation of the solutions

The solutions of MGDG that were used for the measurements of the surface pressure isotherms were prepared by weighing about 0.70–1.00 mg of the lipid on a Cahn electrobalance (Model RG 2000, Ventron Instrument Corp., CA, U.S.A.). The accuracy and precision were within 1%. The balance was constantly flushed with nitrogen during the whole process of the preparation of the solution to prevent contact of the lipid with oxygen. Purified benzene, tested on the monolayer trough as described above, was used as the spreading solvent. The solutions were prepared in a glass vial closed with a special screw-cap mounted with a PTFE Mininert valve (Pierce, Rockford, IL, U.S.A.). This device allows the withdrawal of sample fractions with minimal losses due to evaporation. The molecular weight of MGDG used was 775 g mol^{-1}, calculated from the structural formula, assuming the two side-chains to be α-linolenic acid, the major component of this class of lipids.

RESULTS AND DISCUSSION

The results obtained for the purification of the commercial sample of MGDG are presented in Fig. 1. Curve a shows the pressure–area isotherm obtained for the column chromatographic purification. This curve is the average of four different purifications, the difference between the extremes being about 4 Å^2 per molecule. The extrapolated limiting area per molecule is 92 Å^2. Curve b shows the results obtained for the sample purified by TLC. This curve is the average of eight purifications, the reproducibility being better than 2 Å^2 per molecule. The extrapolated limiting area per molecule in this instance was 82 Å^2. There is therefore a difference of about 10 Å^2 per molecule between the two purification procedures.

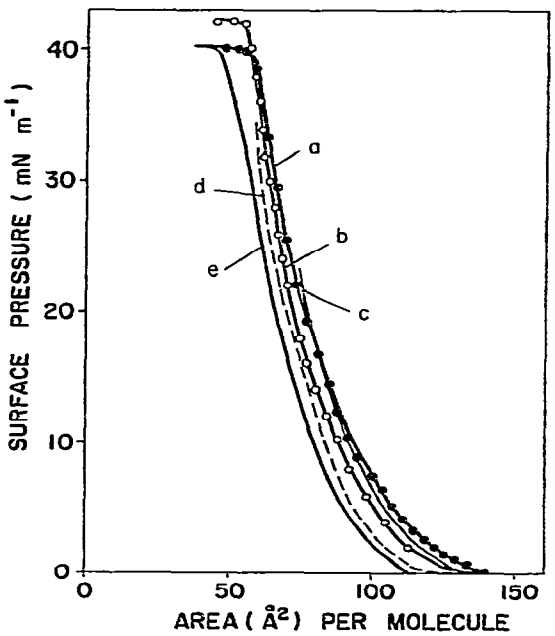

Fig. 1. Pressure–area isotherms for MGDG. (a) Our results for the column chromatography; (b) our results for TLC; (c) results of Liljenberg and Selstam[5,6]; (d) results of Oldani *et al.*[7]; (e) results of Trosper and co-workers[8,9].

The origin of this discrepancy between the two purification techniques lies in the way in which the silica gel is treated during the purification process. The silica gel used for the column chromatography is subjected to drastic conditions, being treated first with sodium ethylate and then with a concentrated solution (20%) of glacial acetic acid in ethanol. Acetic acid has a very strong affinity for silica gel, being very far down in the eluotropic series[4]. The subsequent washings with ethanol and water and the drying of the silica gel in the oven are certainly inadequate to remove all of the acetic acid from the active sites of the silica gel. The presence of the acid on the column obviously has an effect on the purification of the lipid because, as noted above, a faint spot was observed on the TLC plate where the sample was deposited. It is possible that the acid removes the carbohydrate moiety of the galactolipid so

that there are effectively more chains per unit weight. This spot was never observed when the sample was purified by TLC.

In order to test the effect of acetic acid, purification of MGDG by preparative TLC was examined. The purification steps were identical with those described above, except that the plates were washed using chloroform–methanol–acetic acid–water (90:25:15:4) instead of the methanol–water (50:50) normally used. The molecular area of MGDG was found to be 90 $Å^2$ $molecule^{-1}$ (average of three different purifications). Moreover, the TLC pattern of the lipid appeared exactly the same as when it was purified on a column of silica gel treated with acetic acid, the spot where the lipid was deposited being present. These results show that much care has to be taken when acetic acid is used together with silica gel in lipid purification, as degradation of the lipid could occur.

Fig. 1 also shows the comparison between the surface pressure and area per molecule characteristics of MGDG obtained by various workers with respect to the TLC purification we have performed. Liljenberg and Selstam[5,6] published a series of results on the monolayer properties of MGDG. Their results are shown on curve c, the maximum surface pressure attained being 20 mN m^{-1}. The molecular areas are clearly higher than those which were obtained from our TLC purification. Curve d shows the results of Oldani *et al.*[7]. Although the molecular areas can be satisfactorily compared with ours, Oldani *et al.* gave no indication of the collapse pressure obtained with the MGDG sample they used. It has been our experience, mainly with chlorophyll *a*, chlorophyll *b* and phytol, that the collapse pressure could be an indication of the purity of a sample, everything else being the same. The highest collapse pressure was always associated with the purest product as shown by TLC. It would have been interesting in the latter instance to have had this additional information in order to permit a better comparison with our results. Trosper and co-workers[8,9] gave a complete pressure–area curve for MGDG up to the collapse pressure. Their results (curve e) are the average for at least four samples. Although the limiting areas obtained in this instance are similar to ours, the scatter of our results (curve b being the average of eight different purifications, the scatter being less than 2 $Å^2$ per molecule throughout the pressure range) together with the higher collapse pressure that we obtained are a clear indication of the greater degree of purification attained with our technique.

It appears, therefore, that good results can be obtained by using preparative TLC for the ultra-purification of lipids required in monolayer work, provided that precautions are taken at each step of the procedure. Blank experiments on the monolayer trough should be carried out between every step, to ensure that no contamination is introduced. Acetic acid should be used very cautiously or, better, omitted as its strong adsorption on the silica gel can induce degradation. The main advantage of the technique is its efficiency in providing a rapid purification of lipids adequate for surface pressure studies, the procedure taking at most 1 day. It can also be extended to other classes of lipids, phosphatidylcholine having been satisfactorily purified this way in our laboratory[10]. In the latter instance[11], the availability of a well characterized, pure commercial sample of phosphatidylcholine showing only one spot by TLC has demonstrated the validity of our purification technique, the molecular area obtained with the commercial sample being, within experimental error, equal to the molecular area of the lipid purified as described.

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