

Separation of glycosyl diglycerides from phosphatides using silicic acid column chromatography

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SUMMARY A procedure has been developed for the separation of the glycosyl diglycerides from the phosphatides of Gram-positive bacteria on columns of silicic acid. The method utilizes mixtures of acetone in chloroform for elution of the glycosyl diglycerides, followed by increasing amounts of methanol in chloroform for elution of the phosphatides. The course of the fractionation was followed by means of phosphorus and carbohydrate determinations and by paper chromatography. The completeness of the separation of the phosphatides from the sugar-containing lipids was shown also by chromatographing a total lipid extract containing P^{32} -labeled phosphatides.

KEY WORDS phosphatides · glycolipids · bacterial lipids · glycosyl diglycerides · silicic acid chromatography · separation of lipid classes

DIRECT APPLICATION of common silicic acid column chromatography procedures (1-5) to the fractionation of bacterial lipids does not give complete separations. Bacterial lipids in general, and the lipids of Gram-positive bacteria in particular, contain considerable amounts of glycosyl diglycerides (glycolipids) in addition to phosphatides and some neutral lipids. The glycosyl diglycerides and the acidic phosphatides (phosphatidic acid, diphosphatidyl glycerol, and phosphatidyl glycerol) are not separated by the usual silicic acid column procedures. Similar observations have been made with plant lipids, which also are complicated by the presence of glycosyl diglycerides (6-9).

The present paper gives data on the fractionation of lipids from Gram-positive bacteria. A clear-cut separation of glycosyl diglycerides and phosphatides has been achieved by modification of the silicic acid column chromatographic procedure developed in this laboratory (1).

MATERIALS AND METHODS

Growth of Cells

The organisms used in this study were *Streptococcus faecalis* (ATCC 9790) and *Lactobacillus plantarum* B-246. The *L. plantarum* strain was kindly provided by Dr. C. S. Pederson of Cornell University. The composition of the semi-synthetic growth medium (per liter) was as follows: glucose, 20 g; sodium citrate, 20 g; sodium acetate (tri-hydrate), 5 g; NH_4Cl , 3 g; K_2HPO_4 , 5 g; adenine, 10 mg; guanine, 10 mg; uracil, 10 mg; xanthine, 10 mg; thiamine hydrochloride, 1 mg; riboflavin, 1 mg; pyridoxal hydrochloride, 200 μg ; calcium pantothenate, 1 mg; niacin, 1 mg; *p*-aminobenzoic acid, 200 μg ; biotin, 10 μg ; folic acid, 10 μg ; glycine, 0.2 g; L-asparagine, 0.1 g; DL-tryptophan, 0.1 g; L-cystine, 0.1 g; acid hydrolyzed casein (General Biochemicals, Chagrin Falls, Ohio), 5 g; and salts solution,¹ 4 ml. The pH was adjusted to 6.8 with hydrochloric acid.

Extraction by diethyl ether of a 50 g sample of the casein hydrolysate yielded only about 9 mg of a semisolid material, so that the amount of lipoid material introduced into the growth medium by the casein hydrolysate was negligible. To prevent excessive darkening of the medium, the glucose was sterilized separately and then added to the other medium constituents. Six-liter Florence flasks containing 4.5 liters of medium were inoculated with 45 ml of an 18 hr culture grown in the same medium. *S. faecalis* was grown at 37°; *L. plantarum* at 32°. The cell crop was harvested by centrifugation at a time which was well within the phase of exponential growth

¹ Salts solution: $MgSO_4 \cdot 7H_2O$, 100 g; $FeSO_4 \cdot 7H_2O$, 5 g; NaCl, 5 g; $MnSO_4 \cdot 7H_2O$, 2 g, dissolved in 500 ml distilled water.

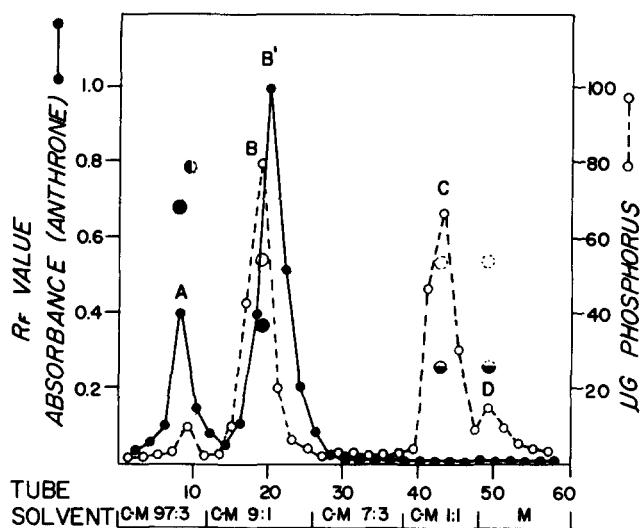


FIG. 1. Column chromatography of a total lipid extract of exponential phase cells of *L. plantarum* on Unisil silicic acid. Elution was with the following solvents in the order given: 60 ml of chloroform-methanol 97:3 (v/v), 70 ml of chloroform-methanol 9:1, 60 ml of chloroform-methanol 7:3, 50 ml of chloroform-methanol 1:1, and 60 ml of absolute methanol. Solvent changes occurred at fractions 12, 26, 38, and 48. R_F values were obtained by chromatography on silicic acid-impregnated paper using diisobutylketone-acetic acid-water 40:20:3 for development. The staining characteristics of the components were: ● yellow color with Rhodamine G and periodate-Schiff positive, ◐ blue color with Rhodamine G, ○ blue color with Rhodamine G and periodate-Schiff positive, ◑ yellow color with Rhodamine G and ninhydrin positive. Weakly staining components are indicated by dashed circles. The left ordinate indicates both the R_F values and the absorbance (anthrone).

unless otherwise specified. The cell paste was washed three times with cold distilled water. Lipid extracts were prepared directly from the washed cells.

Lipid Extraction

In order to establish a reproducible extraction procedure, methanol-diethyl ether 2:1 (v/v) and chloroform-methanol 1:1 (v/v) were compared with chloroform-methanol 2:1 (v/v). In our hands, the most complete extraction procedure involved preliminary heating of the cells with methanol followed by the addition of chloroform to give a final chloroform-methanol concentration of 2:1 (v/v). Preliminary heating with methanol served to split lipid-protein complexes (10), and to inactivate lipases which might be present. Since bacteria contain "bound lipids" several procedures for the extraction of bacterial lipids include extreme treatment of the cells with acids or bases (11). Such treatments may be satisfactory for fatty acid analyses; however, for determining the nature of intact neutral glycerides, phospholipids, and glycosyl diglycerides within the cell, milder extraction procedures are essential. Although a small amount of ether-soluble material was obtained after acid hydrolysis of the extracted cell residue the following extraction pro-

cedure gave consistent and reproducible results with a minimum degradation of lipid material.

All solvents were reagent grade. Chloroform and methanol were redistilled in an all-glass apparatus before use. The ratio of solvent to cell material (wet weight) was 10:1. Methanol was added first and the contents were heated in a water bath at 65° for 5 min. After cooling to room temperature, the chloroform was added and the resulting suspension was stirred at room temperature for 20 min. The cellular residue was removed by filtration through a sintered glass funnel and then reextracted by stirring with fresh solvent at room temperature for 20 min. The filtrates were combined and evaporated in vacuo at 40°. The residue was dried by successive evaporations with benzene-absolute ethanol 4:1 (v/v) and then extracted four times with 15 ml portions of chloroform at 40° for 15 min. The combined chloroform extracts were filtered through a sintered glass funnel. If possible, the lipid extracts were analyzed immediately. If storage was necessary, the chloroform was evaporated under nitrogen and replaced with benzene-absolute ethanol 4:1. Lipid extracts were stored at -20° for not longer than 2 weeks.

Silicic Acid Column Chromatography

A slurry of 10 g of Unisil silicic acid (Clarkson Chemical Co., Williamsport, Pa.) in 75 ml of redistilled *n*-heptane was poured into a glass column, 1.75 cm i.d. The final height of the adsorbent column was 12 cm. It was washed with 60 ml of diethyl ether and with 60 ml of chloroform. The lipid sample, containing 500-600 μ g of phosphorus, was applied to the column in 3-5 ml of chloroform and washed in with two 3-ml portions of chloroform. Neutral lipids were eluted with 60 ml of chloroform and collected as a single fraction. The glycosyl diglycerides were fractionated by use of increasing increments of acetone in chloroform, and the phosphatides by use of increasing amounts of methanol in chloroform (for details see below). Fractions (5 ml) were collected by means of an automatic fraction collector.

Paper Chromatography

Each tube from the column fractionation was analyzed by chromatography on silicic acid-impregnated paper (12). The solvent system for neutral lipids was *n*-heptane-diisobutyl ketone-acetic acid 96:6:0.5 (HDA) and for polar lipids diisobutyl ketone-acetic acid-water 40:20:3 (DAW). Chromatograms were stained with Rhodamine 6G and viewed under ultraviolet light for detection of all lipid components (1, 13). Tests for aminophosphatides, choline, and unsaturation were those outlined by Marinetti (12). Lipids containing vicinal glycols were detected by modification of the procedure of Buchanan et al. (14). Thoroughly dried chromatograms were immersed in

freshly prepared 2% aqueous sodium metaperiodate. The chromatogram was then placed in 2% aqueous sodium bisulfite to remove excess iodate, and then dipped in Schiff's reagent. The chromatogram was allowed to dry at room temperature. The colored spots appeared within an hour. Heating was unnecessary for either the oxidation step or color development.

Analytical Procedures

Aliquots of the column fractions were analyzed for total phosphorus and carbohydrate. Phosphorus was determined by the method of Harris and Popat (15) as modified by Marinetti et al. (16). Assay of carbohydrate was by the anthrone reaction using the procedure of Radin et al. (17).

RESULTS AND DISCUSSION

Figure 1 shows the separation pattern obtained by chromatography of the total lipid from *L. plantarum* using discontinuous elution with chloroform-methanol, a method commonly used to separate animal lipids. A lack of resolution of the glycosyl diglycerides and phosphatides in peak fractions A, B, and B' is shown by the phosphorus and carbohydrate distribution and by paper chromatographic analysis. Changing the ratios of the chloroform-methanol mixtures did not significantly improve

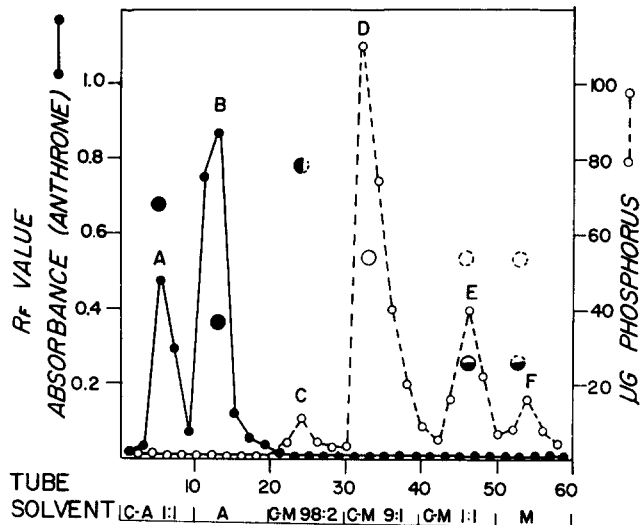


FIG. 2. Column chromatography of a total lipid extract of exponential phase cells of *L. plantarum* on Unisil silicic acid. Elution was with the following solvents in the order given: 50 ml of chloroform-acetone 1:1 (v/v), 50 ml of acetone, 50 ml of chloroform-methanol 98:2, 50 ml of chloroform-methanol 9:1, 50 ml of chloroform-methanol 1:1, and 50 ml of absolute methanol. Solvent changes occurred at fractions 10, 20, 30, 40, and 50. R_F values (indicated by the left ordinate) were obtained by chromatography on silicic acid-impregnated paper using diisobutylketone-acetic acid-water 40:20:3. The staining characteristics of the components were as described for Fig. 1. The left ordinate indicates both the R_F value and the absorbance (anthrone).

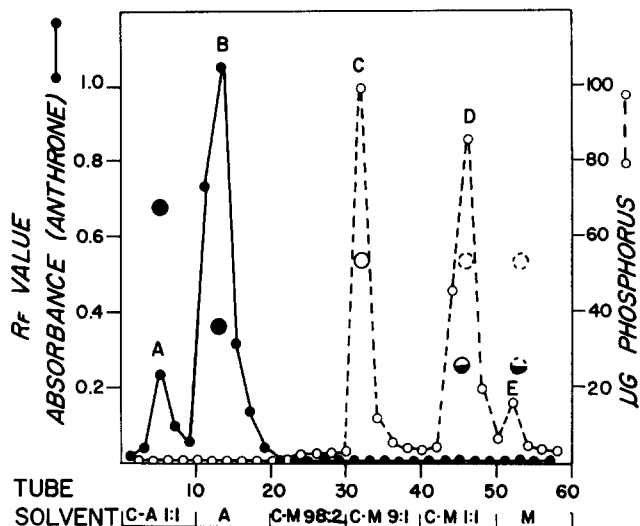


FIG. 3. Column chromatography of a total lipid extract of stationary phase cells of *S. faecalis* on Unisil silicic acid. The solvent mixtures used for elution of the various components and all other details as in Fig. 2.

the separation. A similar elution pattern was observed with the total lipid extract obtained from *S. faecalis*.

Since separation of the glycosyl diglycerides from the acidic phosphatides was prerequisite to structural studies, other solvents were evaluated. It was found that acetone would elute only neutral lipids and glycosyl diglycerides from columns of Unisil silicic acid. Either chloroform or diethyl ether would elute neutral lipids but not the glycosyl diglycerides or phosphatides. Mixtures of acetone and diethyl ether will elute glycosyl diglycerides from silicic acid columns but some phospholipid also is eluted. Mixtures of chloroform and acetone, however, elute only glycosyl diglycerides.

Figure 2 illustrates a typical separation of glycosyl diglycerides and phosphatides obtained by incorporating acetone into the solvent mixture. The lipid extract was prepared from *L. plantarum*. Nonpolar lipids were eluted first with chloroform alone and were collected as a single fraction. Analysis of the fractions for phosphorus and carbohydrate as well as paper chromatography showed complete separation of the glycosyl diglycerides from the phosphatides. The peak fractions A and B contained the glycosyl diglycerides and were eluted with increasing concentrations of acetone in chloroform. The phosphatides, found in peak fractions C, D, and E, were eluted with 2%, 10%, and 50% methanol in chloroform, respectively. The last peak, F, eluted with absolute methanol, may be an artifact.

Fractionation of a total lipid extract prepared from stationary phase cells of *S. faecalis* is shown in Fig. 3. As observed with *L. plantarum* lipids, the glycosyl diglycerides are completely separated from the acidic phosphatides.

The efficiency of the column procedure for separating glycosyl diglycerides and the acidic phosphatides was determined further by chromatographing a total lipid extract prepared from *S. faecalis* cells which were grown in the presence of orthophosphate- P^{32} . The acetone eluates contained only 0.3% of the total radioactivity of the lipid applied to the column, the remaining radioactivity being found in the methanol eluate.

Separation of glycosyl diglycerides from the acidic phosphatides is of importance since these two classes of lipids constitute the major polar lipids of many Gram-positive bacteria and also occur together in plants. Data concerning the structure of lipids fractionated by this procedure will be published later.

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