Separation of glycosyl diglycerides from phosphatides using silicic acid column chromatography

MARIE L. VORBECK and G. V. MARINETTI
Department of Biochemistry, The University of Rochester, School of Medicine and Dentistry, Rochester, New York

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 P4 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, diphasphatidyl 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 semisynthetic growth medium (per liter) was as follows: glucose, 20 g; sodium citrate, 20 g; sodium acetate (trihydrate), 5 g; NH4Cl, 3 g; KH2PO4, 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, 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: MgSO4·7H2O, 100 g; FeSO4·7H2O, 5 g; NaCl, 5 g; MnSO4·7H2O, 2 g, dissolved in 500 ml distilled water.
bound lipids" several procedures for the extraction of
with Rhodamine G and periodate-Schiff positive,
methanol 1
silicic acid-impregnated paper using diisobutylketone-acetic acid-
form to give a final chloroform-methanol concentration
extraction procedure involved preliminary heating of the
cells with methanol followed by the addition of chloro-
lipases which might be present. Since bacteria contain
4
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 meth-
anol were redistilled in 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 evap-
orations with benzene–absolute ethanol 4:1 (v/v) and
then extracted four times with 15 ml portions of chloro-
form at 40° for 15 min. The combined chloroform ex-
tracts were filtered through a sintered glass funnel. If pos-
sible, 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 µg 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 frac-
tionated 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 Mari-
netti (12). Lipids containing vicinal glycols were detected
by modification of the procedure of Buchanan et al. (14).
Thoroughly dried chromatograms were immersed in

**Fig. 1.** Column chromatography of a total lipid extract of expo-
nential 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 46. *R*<sub>F</sub> 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 perio-
date–Schiff positive, : blue color with Rhodamine G, : blue color
with Rhodamine G and periodate–Schiff positive, : blue color
with Rhodamine G and ninhydrin positive. Weakly staining compo-
nents are indicated by dashed circles. The left ordinate indi-
cates both the *R*<sub>F</sub> values and the absorbance (anthrone).

unles 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 chloro-
form to give a final chloroform–methanol concentration
of 2:1 (v/v). Preliminary heating with methanol served
to splip 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 satisfac-
tory 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
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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. 1](image_url)

**Fig. 1.** Column chromatography of a total lipid extract of exponential phase cells of *L. plantarum* 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.

![FIG. 2](image_url)

**Fig. 2.** Column chromatography of a total lipid extract of exponential phase cells of *L. plantarum* on Unisil silicic acid. The solvent mixtures used for elution of the various components and all other details as in Fig. 1. The left ordinates indicates both the *Rf* value and the absorbance (anthrone).

![FIG. 3](image_url)

**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 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-P32. 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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**References**