PHOSPHOLIPIDS FROM THE CHEMOAUTOTROPH FERROBACILLUS FERROOXIDANS

M.S. Korczynski, A.D. Agate and D.G. Lundgren
Biological Research Laboratories
Department of Bacteriology and Botany
Syracuse University, Syracuse, New York

.Received August 28, 1967

The chemoautotroph Ferrobacillus ferrooxidans like other gram-negative bacteria possesses a multi-layered cell envelope (Remsen & Lundgren 1966) which is believed to be involved in Fe^{++} ion oxidation - the primary energy yielding reaction of the organism (Dugan and Lundgren, 1965). This report presents a phase of our study concerning the characterization of phospholipids in membranes of F. ferrooxidans. Reports of phospholipids in other chemoautotrophs have been published (Schaeffer and Umbreit, 1962; Jones and Benson, 1965; Hagen, Goldfine, and Williams, 1966).

EXPERIMENTAL METHODS

Ferrobacillus ferrooxidans was cultured as described by Silverman and Lundgren (1959), and cells were harvested during early stationary phase (48 to 54 hr) using a Sharples centrifuge. The cell paste was washed three times in β-alanine sulfate buffer (.01M, pH 3.6) in a refrigerated centrifuge at 10,000 x g for 15 min prior to lipid extractions.

Initially, total cell lipid was determined using a modified method of Kates (1965). 300-500 mg wet wt. of whole cells were treated with $CH_3OH:CHCl_3$ 2:1 (v/v) for 24 hr, centrifuged at 10,000 x g for 15 min, and the cell residue retreated for an additional 18-21 hr with $CH_3OH:CHCl_3:H_2O$ 2:1:0.8 (v/v). The extracted cells were again centrifuged and the two solvent volumes pooled and taken to dryness under N_2. The total lipid content was determined by weighing and constituted 19-21% of the total dry weight of the cells.

For lipid fraction extraction and subsequent analysis a procedure consisting of the combined methods of Macfarlane (1961) and Kates (1965) was used (Fig. 1).

Following lipid extraction, 2-3 mg of lipid I and II were placed on individual silicic acid columns prepared according to the procedure of Mehlman et al. (1966) and separated into neutral and phospholipid fractions. Neutral lipids were eluted with 35-40 ml of $CHCl_3$ while phospholipids were eluted with the same volume of CH_3OH. Neutral and phospholipid fractions from lipid I and II were weighed after drying under N_2.

The separated phospholipid fractions were chromatographed on SG.81 silica gel impregnated paper (Wuthier, 1966), and P.81 cellulose phosphate paper (Marinetti, 1962). Chromatograms were stained with ninhydrin and also with iodine vapors.

Spots on chromatograms were cut out and heated at 180°C in 2 ml of 10 N H_2SO_4 for 1 hr in sealed test tubes to release esterified phosphate. Phosphorus was determined by the method of Taussky and Shorr (1953). Both neutral and phospholipid fractions obtained after column chromatography were assayed for Mg^{++} by atomic absorption spectrophotometry.

The phospholipids were deacylated by alkaline hydrolysis and the water soluble components chromatographed on paper (Dawson, 1960). Fatty acid methyl esters of the alkaline hydrolyzed phospholipids were prepared by refluxing with boron trifluoride in methanol, and a qualitative evaluation of the methylated fatty acid esters was made using GLC and methylated standards.

RESULTS

Table 1 shows the percent of phospholipid and neutral lipid of lipid I and II fractions of F. ferrooxidans. Results represent averages of four separate determinations on different cell batches.

DISCUSSION

We believe that lipid I is structurally related to the outer membrane of the cell envelope as seen in thin sections and in freeze-etched cells. (see Figs. 1, 2, and 4, J. Bacteriol. 92:1765-1771). Evidence for this belief will be described elsewhere but is based on electron micrographs of envelope surfaces after chemical

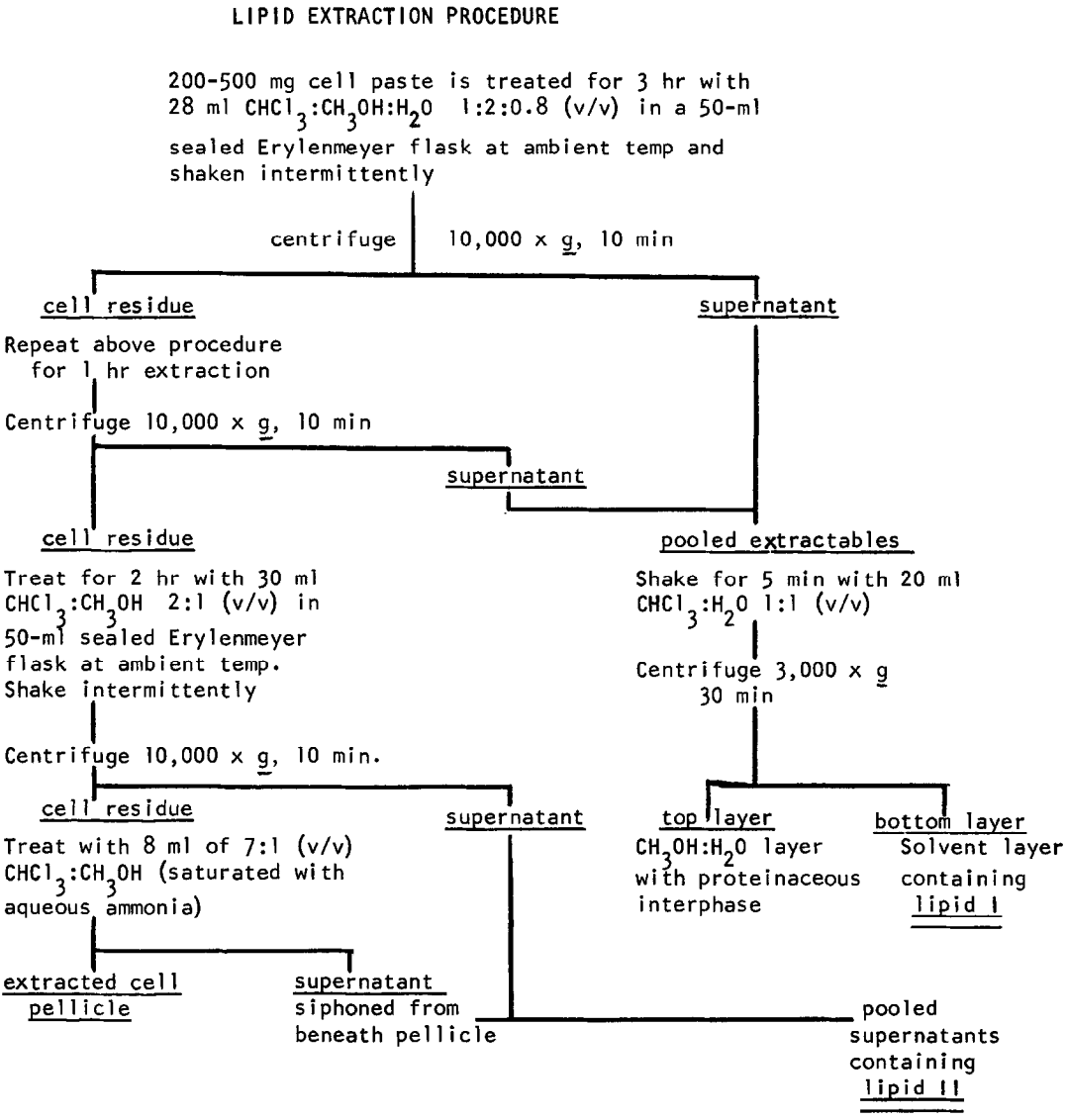

Fig. 1. Flow diagram of the extraction procedure for the differentiation of loosely bound lipid I and tightly bound lipid II.

---

and enzyme treatment.

The relatively large amount of PS in the outer layer may aid the organism in maintaining a negatively charged cell envelope to facilitate Fe^{++} binding prior to substrate oxidation. The high level of PS in Ferrobacillus is not typical of bacteria (Ikawa, 1967) and suggests some special function as mentioned above.

Lipid II was considered more tightly bound for it was removed only after using a higher ratio of chloroform to methanol and extending the extraction time. This may indicate that lipid II is a component of the inner layers of the wall or of the cytoplasmic membrane. Analyses show the phospholipid of lipid II to be a lyso derivative of either PS or PE which forms during chemical extraction and would not be found in vivo. Thus the similarity in the phospholipids of the two fractions would agree in part with the report of Hancock and Meadow (1967) stating that the lipoprotein layer of the cell wall and the cytoplasmic membrane in a Gram-negative Pseudomonas contain almost identical lipids.

The fact that three times more Mg^{++} was associated with phospholipids of lipid II suggests a more tightly bound association of the cation with lipids of the cytomembranes. Gordon and MacLeod (1966) found Mg^{++} associated with envelope diphosphatidyl glycerol in Pseudomonas spp.

Lipid II contains about 3 times the neutral lipid of lipid I. The significance of this is unknown. We have yet to identify the neutral lipids or the protein interphase (Fig. 1).

TABLE 1. Percent of phospholipid and neutral lipid of Ferrobacillus ferrooxidans separated on silicic acid columns and assayed gravimetrically.

LIPID FRACTION	LIPID I Avg.	LIPID II Avg.
Phospholipid	79 ± 1%	28 ± 0.5%
Neutral lipid	26 ± 2	76.8 ± 1.8
% of total lipid	54.2 ± 1.2	45.8 ± 1.2
% of cell dry wt	11 ± 1	9 ± 1

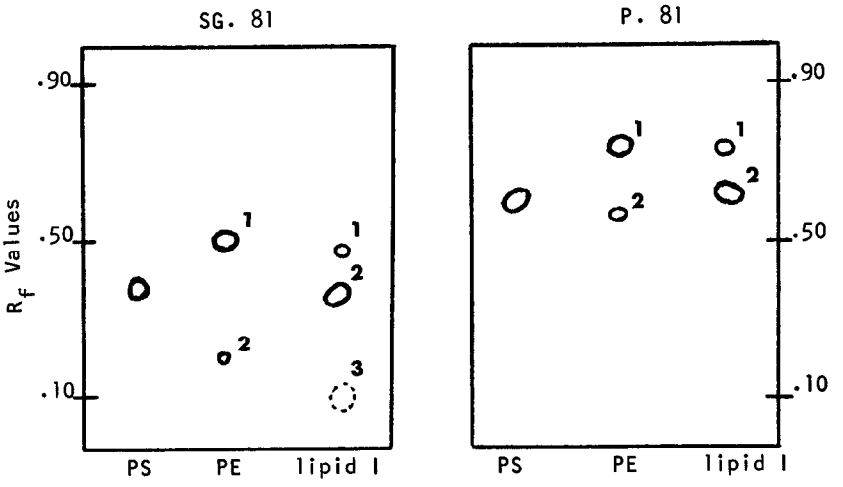

Fig. 2 Tracings of ascending chromatograms of standards and lipid I phospholipids. The solvents for SG.81 and P.81 paper were $CHCl_3$:CH_3OH:CH_3COOH: diisobutyl ketone (DIBK):H_2O:23:10:25:45:4 (v/v) and DIBK: CH_3COOH:H_2O 40:20:3 (v/v), respectively.

The phospholipid fraction of lipid I showed two ninhydrin positive spots with an occasional appearance of a third spot (fig. 2). The major component in lipid I migrated with an R_f value comparable to that of phosphatidylserine (PS) and it co-chromatogramed with the standard. The minor component migrated as phosphatidylethanolamine (PE). Chromatograms of the PE standard repeatedly showed two components where the lower one was shown to be a lyso-PE derivative (Marinetti, 1961).

Chromatograms of phospholipid from lipid II showed a single ninhydrin and iodine staining component identical to the occasional appearing component 3 of lipid I. This component was shown to be a lyso-derivative of either PS or PE based upon chromatograms of water soluble products after mild alkaline and acid hydrolysis of phospholipid fractions (Dawson, 1960). Paper chromatograms of the products consistently showed ninhydrin staining glycerylphosphatides (water soluble products) with similar R_f values.

The phospholipid fraction of lipid I and II had a phosphorus ratio of 3.66:1. PS represented about 49% of the total phospholipid phosphorus while PE accounted

for about 19%. When the third component of lipid I was detected, it represented 5-24% of the total phospholipid phosphorus. Mg^{++} was found associated only with lipid I and II phospholipid. The ratio of Mg^{++} of lipid I:II was 1:3.45 (μgm Mg^{++}/mg lipid). Identical fatty acids in lipid I and II phospholipid were found with C_{12}, C_{14}, C_{15} and C_{16} fatty acids the most abundant and smaller amounts of C_{17}, C_{18}, and C_{16}, C_{18} unsaturated acids were detected. An unidentifiable fatty acid (probably a C_6 fatty acid) was also detected in both phospholipid fractions.

ACKNOWLEDGEMENT

This work was supported in part by a NIH Training Grant No. 5 T01 GM 00991-03 and by Grant WP00548-05 from the Federal Water Pollution Control Administration, Dept. of the Interior. The technical assistance of Mr. Wayne Crosby and Mr. Arnold Miller is acknowledged.

REFERENCES

Dawson, R.M.C., 1960. Biochem. J. 75, 45.
Dugan, P.R., and Lundgren, D.G., 1965. J. Bacteriol., 89, 825.
Gordon, I.C., and MacLeod, R. A., 1966. BBRC 24, 684.
Hagen, P.O., Goldfine, H., and Williams, P.J. Le B. 1966. Science 156, 1543.
Hancock, I.C., and Meadow, P.M., 1967. J. Gen. Microbiol. 46, x.
Ikawa, M., 1967. Bacterial. Rev., 31, 54.
Jones, G.E., and Benson, A.A., 1965. J. Bacteriol., 89, 260.
Kates, M., Yengoyan, L.S., and Sastry, P.S., 1965. Biochim. Biophys. Acta 98, 252.
Macfarlane, M.G., 1961. Biochem. J., 78, 44.
Marinetti, G.V., 1962. J. Lipid Res. 3, 1.
Mehlman, M.A., Therriault, D.G., Porter, W., Stoewsand, G.S., and Dymsza, M.A., 1966. Jour. Nutrition, 88, 215.
Schaeffer, W.I., and Umbreit, W.W., 1963. J. Bacteriol., 85, 492.
Remsen, C., and Lundgren, D.G., 1966. J. Bacteriol., 92, 1765.
Silverman, M.P., and Lundgren, D.G., 1959. J. Bacteriol., 77, 642.
Taussky, M.A., and Shorr, E., 1953. J. Biol. Chem., 202, 675.
Wuthier, R.E., 1966. J. Lip. Res., 7, 544.