

## BBA Report

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### CARDIOLIPIN, A MAJOR PHOSPHOLIPID OF GRAM-POSITIVE BACTERIA THAT IS NOT READILY EXTRACTABLE

M.H. FILGUEIRAS and J.A.F. OP DEN KAMP\*

*Biochemisch Laboratorium, Rijksuniversiteit Utrecht, Padualaan 8, 3584 CH Utrecht (The Netherlands)*

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Extraction of phospholipids from stationary phase grown cells of the Gram<sup>+</sup> bacteria, *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus cereus* and *Micrococcus lysodeikticus* was found to be incomplete with various commonly used extraction procedures. Phosphatidylglycerol and phosphatidylethanolamine were readily extracted but up to 95% of the cardiolipin appeared to be retained within the cell residue. Extraction of the cardiolipin could be slightly enhanced by increasing the temperature or the acidity of the extraction solutions but complete extraction was obtained only after lysozyme treatment of intact cells or cell residues remaining after extraction. In addition complete extraction could be observed in the case of cells harvested in the early logarithmic phase. Freeze-fracture electron microscopy was carried out on the cell residue remaining after extraction of all phospholipids except cardiolipin. A fracture plane through the plasma membrane could not be observed anymore. Instead fracture planes through lipid vesicles were observed. These vesicles reside within the remnants of the cytoplasm and consist most likely of the non-extracted cardiolipin.

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The cytoplasmic membranes of Gram<sup>+</sup> bacteria contain cardiolipin, phosphatidylglycerol and, in some organisms, phosphatidylethanolamine as the major phospholipids [1]. The relative amounts in which these phospholipids occur, vary from one organism to another. In addition, substantial variations in phospholipid composition have been reported for a single organism. In part, this can be ascribed to different growth conditions (medium, pH, temperature, growth phase) and furthermore strain differences can be involved [2]. However, some of the reported phospholipid compositions

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\*To whom correspondence should be addressed.

TABLE I

EXTRACTION OF PHOSPHOLIPIDS FROM VARIOUS GRAM<sup>+</sup> BACTERIA

Cells and protoplasts were obtained as described in the text. The cell residue remaining after a Bligh and Dyer extraction was dried under nitrogen, suspended in buffer, treated with lysozyme as described in the text and extracted once more. The data are expressed as percentages of total [<sup>32</sup>P]phospholipid present in lipid extracts from protoplasts. It is assumed that all of the phospholipid is extracted from protoplasts.

	Protoplasts	Whole cells	Cell residue after lysozyme treatment
<i>B. megaterium</i>	100	65	35
<i>B. subtilis</i>	100	60	40
<i>B. cereus</i>	100	87	13
<i>M. lysodeikticus</i>	100	61	39

differ in an extreme way which can best be illustrated by a short summary of the data obtained with *B. megaterium*. The published values for the cardioli-  
pin content of *B. megaterium* vary from a few percent [3–6] to 12% [7] and even up to 57% [8]. During a recent study on the phospholipid local-  
ization in the membrane of *B. megaterium* [9] we used the same strain as  
before [3] but found a cardioli-  
pin content of 45% instead of a few percent. The explanation for these discrepancies is offered by the data presented  
here. In all cases in which high amounts of cardioli-  
pin were found, lipid ex-  
traction was carried out after removal of the cell wall whereas extraction of  
whole cells results in a low recovery of cardioli-  
pin.

*B. megaterium* (MK 10D), *B. subtilis* (strain Marburg) and non-identified  
strains of *B. cereus* and *M. lysodeikticus* were grown under continuous  
aeration at 37° C in a medium containing 1% peptone, 1% yeast extract, 0.5%  
NaCl and 0.04% Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2. Routinely 300 μCi [<sup>32</sup>P]phosphate was  
added per l medium. Cells were harvested after 16 h of growth by centri-  
fugation, washed with 10 mM K-phosphate buffer, pH 7.5, and suspended in  
the same buffer. For the preparation of protoplasts, cells were suspended in  
this buffer containing in addition 15 mM MgCl<sub>2</sub> and 0.4 M sucrose. Lyso-

TABLE II

NATURE OF PHOSPHOLIPIDS EXTRACTED FROM WHOLE CELLS, PROTOPLASTS AND CELL  
RESIDUE FROM *B. MEGATERIUM*

Lipid extracts were analyzed by thin-layer chromatography as described before [9]. Spots were visu-  
alized with I<sub>2</sub>, scraped off and <sup>32</sup>P was counted in a Packard liquid scintillation counter. Data are ex-  
pressed as percentages of the total [<sup>32</sup>P]phospholipid present in lipid extracts from protoplasts. Some  
discrepancy is noted between total phosphatidylglycerol and cardioli-  
pin contents, which is due to a  
gradual conversion of phosphatidylglycerol into cardioli-  
pin during protoplast formation. The other  
phospholipid fraction mainly consists of glucosaminylphosphatidylglycerol.

	Protoplasts	Whole cells	Cell residue after lysozyme treatment
Total phospholipid	100	65	35
Cardioli- pin	40	4	33
Phosphatidylglycerol	40	41	2
Phosphatidylethanolamine	16	16	±
Other	4	4	±

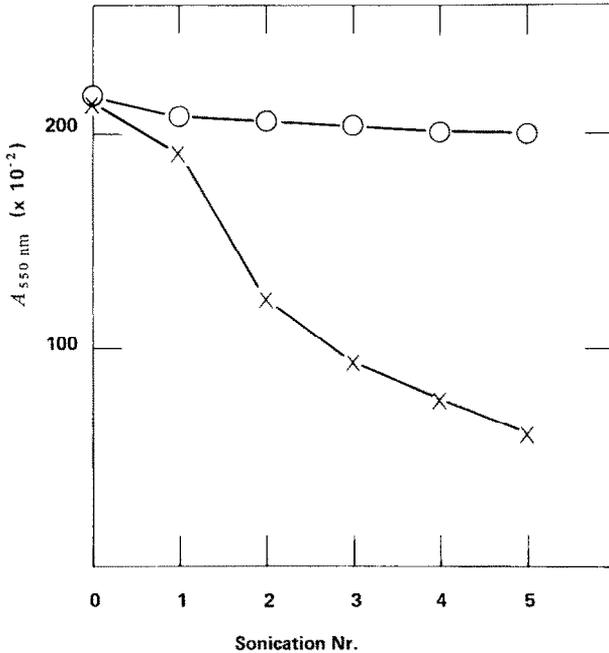


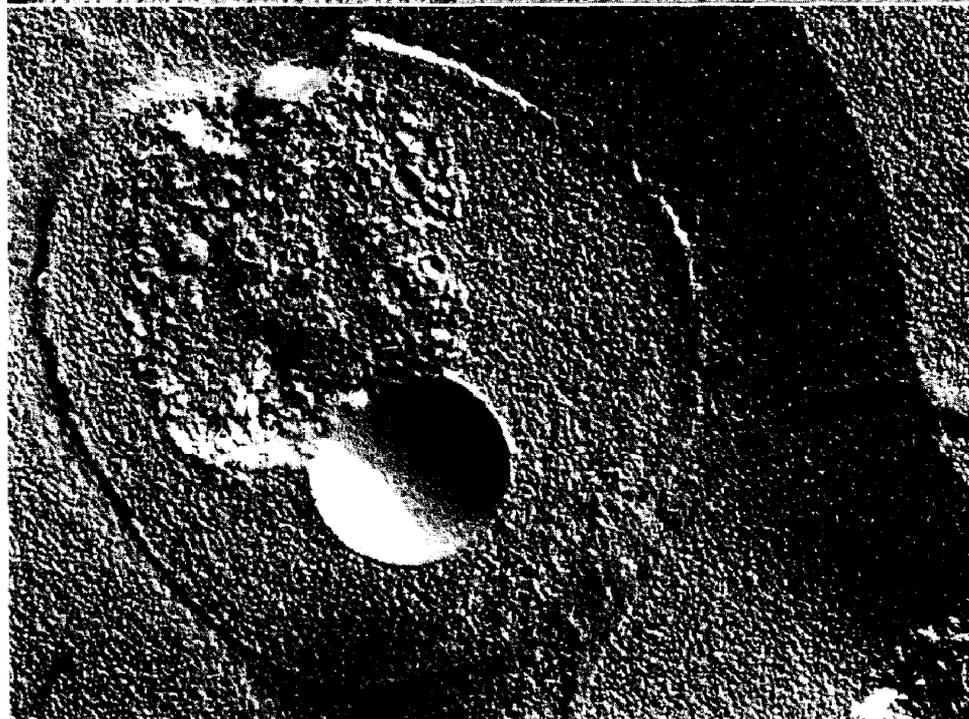
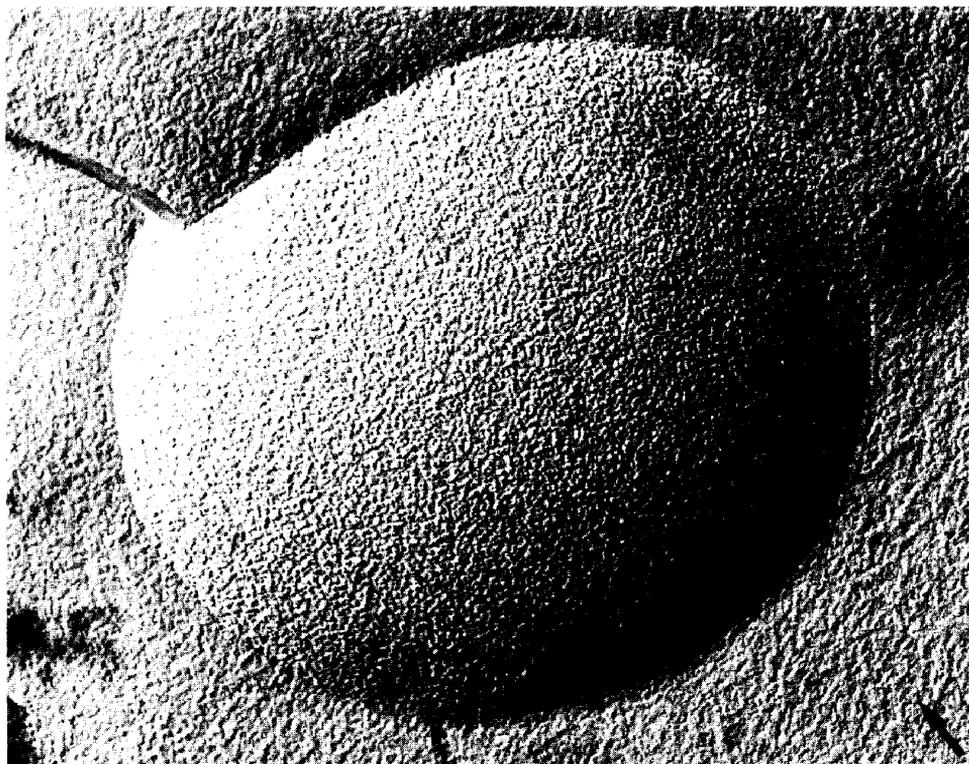
Fig. 1. Destruction of *B. megaterium* cells by ultrasonication. Cells were harvested from the early logarithmic growth phase ( $A_{550 \text{ nm}} = 0.360$ ) (X) and at the end of the logarithmic growth (O). The cells were suspended in the phosphate buffer to  $A = 0.210$  at 550 nm. Ultrasonication was carried out with a Branson sonifier (60 W output) in 30-s pulses with 1 min intervals. Samples were cooled in ice throughout. The figure shows the stability of cells harvested at the end of the logarithmic phase (O) and the rapid disintegration of cells harvested at the early growth stage (X).

zyme (4.2 mg/ml) was added and incubation at 37°C was performed until protoplast formation was complete as judged by phase-contrast microscopy.

A lipid extraction according to Bligh and Dyer [10] was carried out on whole cells and protoplasts of the four organisms. The results are summarized in Table I. Extraction is incomplete when whole cells are extracted. The residual phospholipid appears to be present in the cell residue which remains after the Bligh and Dyer extraction and can be dissolved only after lysozyme treatment of this residue. Identical results were obtained using the extraction procedures described by Folch [11] and Rose and Oklander [12].

The nature of the phospholipid which is retained within the cell residue was investigated by thin-layer chromatography [9] of lipid extracts obtained after lysozyme treatment of this residue. The results for *B. megaterium* are shown in Table II. It is obvious that cardiolipin is not extracted as long as the cell wall is present. Similar data were obtained with the other organisms under study (data not shown). One can envisage the possibility that cardiolipin is retained within the cell by a strong interaction with some intracellular constituents. To investigate this, the following experiments were car-

Fig. 2. Freeze-fracture electron microscopy of *B. megaterium*. Cells of *B. megaterium* were studied using freeze-fracture electron microscopy before (top) and after (bottom) lipid extraction. Before lipid extraction a fracture plane is observed typical for plasma membranes. The plasma membrane structure is lost during extraction; instead lipid vesicles (v) and remnants of the cell wall (w) and cytoplasm (c) can be seen. Magnification,  $\times 100\ 000$ . Arrows indicate the directions of shadowing.



ried out. The cell residue remaining after one extraction step was dried, resuspended in buffer and incubated for 1 h at 37°C with one of the following compounds: EDTA (up to 50 mM); EGTA (up to 50 mM); toluene (10%); deoxycholate (0.05–0.4%); sodium dodecyl sulfate (0.05%); trypsin (1 mg/ml); pronase (1 mg/ml); chymotrypsin (1 mg/ml); RNAase (1 mg/ml); DNAase (1 mg/ml) and various combinations of detergents and enzymes listed above. Then the residue was extracted for a second time but none of the entrapped cardiolipin could be recovered. It is indicated by these results that mucopeptide-teichoic acid layer forms the only barrier for cardiolipin extraction. This possibility is underlined by the following experiment. Cells, harvested in the early logarithmic growth phase ( $A_{550 \text{ nm}} = 0.360$ ) were compared with cells harvested at the end of the logarithmic growth ( $A = 2.350$ ). The former cells appeared to be surrounded by a thin cell wall as seen in electron microscopy (data not shown) and were fragile. Ultrasonication resulted in a rapid destruction of the cells (Fig. 1). Bligh and Dyer extraction of these cells resulted in complete extraction of all cardiolipin present. In contrast full grown cells did have a thick cell wall, were much more resistant against sonication and appeared to retain all of their cardiolipin within the cell during extraction. How such a barrier function of the cell wall is established is not known. However it can be speculated that the thick, highly polar, negatively charged layer prevents an effective penetration of the chloroform. This may result in a chloroform concentration within the cell which is too low to solubilize cardiolipin. The low solubility of this as well as other highly negatively charged phospholipids has been noted before [13]. The presence of acid in the extraction medium is known to increase the solubility of these phospholipids [13]. A similar observation was made in our studies. Extraction of whole cells or cell residues in the presence of 0.1 M HCl or acetic acid slightly enhanced the recovery of cardiolipin. Up to 20% of the total amount could be solubilized this way. That the low solubility of cardiolipin is responsible for its retention is shown furthermore by the observation that an increase in temperature at which the extraction is performed yields higher amounts of cardiolipin. At 45°C up to 50% can be extracted.

During extraction of whole cells the membrane structure is lost. That can clearly be visualized by freeze-fracture electron microscopy. The normally observed fracture plane through the plasma membrane (Fig. 2, top) is no longer seen; instead fracturing occurs through the cell interior. The cell wall contour as well as the remnants of the cytoplasm are clearly visible after lipid extraction (Fig. 2, bottom). In addition lipid vesicles can be observed adhering to or embedded in the cytoplasm. Most likely the non-extracted cardiolipin is present in these vesicles.

The data presented above explain the discrepancies which exist concerning the actual cardiolipin content of Gram<sup>+</sup> bacteria. Extraction of whole cells, when harvested from the late logarithmic or stationary phase, yields low recoveries of cardiolipin [3–7]. The true cardiolipin content can be estimated only after rupture of the cell wall. These observations are relevant in view of recent interests in the mechanisms of phospholipid biosynthesis in

these organisms [5, 6]. It is feasible that cardiolipin is a major phospholipid also in the strains of *B. megaterium* used in these experiments. If so, a number of observations, especially those regarding the high turnover of phosphatidylglycerol, which is considered to be the direct precursor of cardiolipin [14] might be easier to understand.

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