# The interaction of *Bacillus* protoplasts with sonicated phosphatidylcholine liposomes

M.A. Urbaneja, A. Villena and F.M. Goñi

*Department of Biochemistry, Faculty of Science, PO Box 644, Bilbao, Spain*

Received 31 January 1984

When protoplasts from *Bacillus subtilis* are incubated with sonicated liposomes made from egg-yolk phosphatidylcholine, this phospholipid is incorporated into the protoplast membranes. Biochemical, fluorescence and ultrastructural data suggest that incorporation occurs through membrane fusion.

Bacillus *Protoplast* *Liposome* *Membrane fusion*

## 1. INTRODUCTION

Liposome–cell interactions are of much interest because of the potential use of liposomes as drug carriers or vectors of genetic information. The interaction of mammalian cells with liposomes has been studied in detail, but bacteria have not been sufficiently explored in this respect [1–3].

Among bacteria, *Bacillus* is often used for cloning specific genes [4]; therefore, it is worth considering the possible interactions between liposomes and *Bacillus* protoplasts. Protoplasts are required to facilitate membrane-membrane contact; they can be easily obtained from the whole bacterium, and the cell regenerates readily [5].

We have here studied the interaction between *Bacillus* protoplasts and liposomes made from egg-yolk phosphatidylcholine. This phospholipid has the advantage of inexpensive and easy preparation and a great tendency to form closed vesicles. In addition, *Bacillus* membranes lack phosphatidylcholine, and thus any incorporation from liposomal to protoplast membranes is easily detected.

## 2. MATERIALS AND METHODS

*B. subtilis* 168 $T^+$ was strain no.461 from the Colección Española de Cultivos Tipo. Cells were grown in a medium containing 0.9% bacteriological peptone (Merck), 0.9% yeast extract (Oxoid), 0.45% NaCl, 0.04% $Na_2PO_4$, 2% glucose (pH 7.2). Growth took place in 250-ml flasks containing 50 ml medium at 37°C with orbital shaking at 100 rpm. Cells were harvested by centrifugation at 12000 × *g* for 15 min, and washed in buffer A (10 mM Tris–HCl, 0.4 M sucrose, 0.015 M $MgCl_2$, pH 7.5). Protoplasts were prepared by resuspending the cells in buffer A (2 mg cell dry wt/ml); lysozyme (1.5 mg/ml) was then added, and the suspension incubated in a shaking water bath at 37°C and 100 rpm for 1 h. Protoplast formation was checked by direct observation under a phase-contrast microscope. Protoplasts were obtained by centrifuging the suspension at 12000 × *g* for 15 min, and washed twice with buffer A.

Egg-yolk phosphatidylcholine was purified as in [6]. Liposomes (0.9 mg lipid/ml) were prepared in buffer A and sonicated in an MSE sonicator at 10–12 $\mu$m amplitude for 15 min.

Thirty ml of a protoplast suspension giving an absorbance of 0.4 at 600 nm (corresponding to about 7.5 × $10^8$ protoplasts/ml) were mixed with 2 ml of the sonicated liposome suspension, and the mixture incubated at room temperature for various times between 1 and 60 min. After incubation, the protoplasts were recovered by centrifugation at 12000 × *g* for 15 min (the sonicated liposomes did



*Published by Elsevier Science Publishers B.V.*

not sediment under these conditions) and washed 3 times in buffer L (0.01 M Tris–HCl, 0.19 KCl, 0.015 M $MgCl_2$, pH 7.5). The high ionic strength of this buffer was useful for removing phospholipid vesicles electrostatically bound to protoplasts.

When required, protoplasts were incubated with liposomes in the presence of 0.01 M $NaN_3$, NaF or KCN. In some experiments, incubated protoplasts were treated with trypsin (Sigma type II) (0.01%, w/v, in buffer A) at 37°C for 10 min. The reaction was stopped with Sigma trypsin inhibitor at 0.1% final concentration.

Lipids were extracted from protoplasts as in [7] and separated on silica gel H plates with chloroform–methanol–water (65:25:4, by vol.). Lipid P was determined as in [8], and proteins estimated as in [9].

6-Carboxyfluorescein or phosphotungstate was trapped in liposomes as in [10]. Fluorescence was excited at 490 nm; emission was observed at 520 nm. Fluorescence measurements were carried out in a Perkin-Elmer MPF-3 spectrofluorimeter.

## 3. RESULTS

When *Bacillus* protoplasts are incubated with sonicated phosphatidylcholine liposomes, this phospholipid is readily incorporated into the protoplasts. Significant incorporation occurs after 1 min incubation, and is maximal after 30 min (fig.1a). Phosphatidylcholine incorporation clearly produces an increase in the lipid P/protein ratio of protoplast membranes (fig.1b). Nevertheless, after 60 min incubation, the lipid P/protein ratio decreases again.

When protoplasts are incubated with liposomes in the presence of various metabolic inhibitors (KCN, NaF, $NaN_3$), phosphatidylcholine incorporation is not decreased as judged from the percent phospholipid distribution of treated protoplasts (table 1). However, KCN and $NaN_3$ result in lysis of 39 and 23% of the protoplasts, respectively, as measured by turbidity and cell counting; no such effect is seen with NaF.

In a series of experiments, protoplasts incubated with liposomes and washed with buffer L were treated with trypsin. Trypsin is active in cleaving protoplast proteins: in control (non-incubated) protoplasts, trypsin produces an increase of about

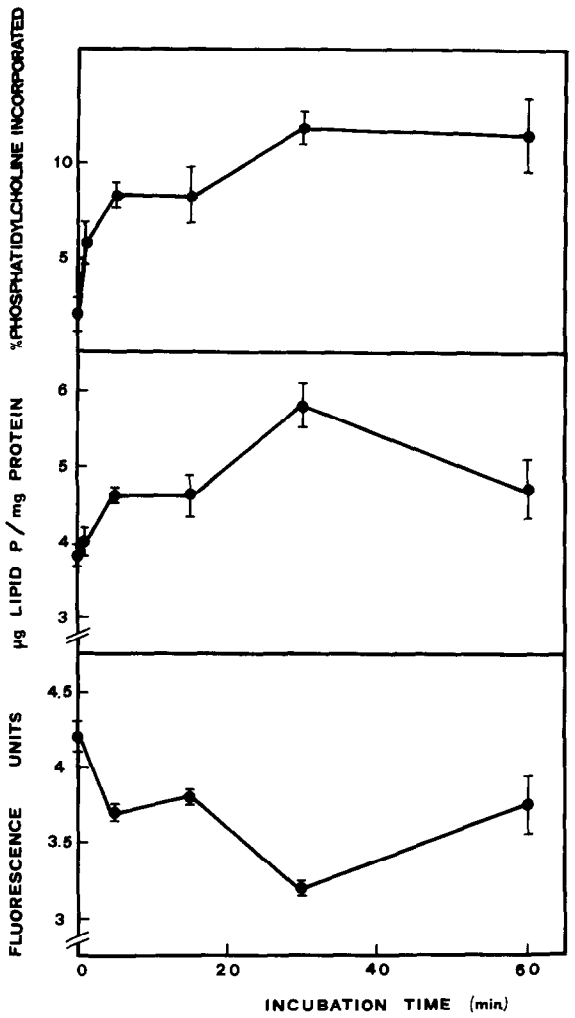


Fig.1. (a, top) The proportion of phosphatidylcholine (expressed as % of total lipid P) in *Bacillus* protoplast membranes, as a function of time of incubation with phosphatidylcholine liposomes. Zero time corresponds to protoplasts not incubated with liposomes. (b, middle) Phospholipid/protein ratio (expressed as µg lipid P/mg protein) of *Bacillus* protoplasts, as a function of time of incubation with phosphatidylcholine liposomes. Zero time corresponds to protoplasts not incubated with liposomes. (c, bottom) Fluorescence (arbitrary units) of 6-carboxyfluorescein as a function of time of protoplast and liposome incubation. Zero time corresponds to liposomes not incubated with protoplasts. Data correspond to mean values ± SE of 6 experiments.

65% in the lipid P/protein ratio: from 3.8 to 6.3 µg lipid P/mg protein. However, trypsin treatment does not decrease the amount of protoplast-

Table 1

The effect of trypsin and metabolic inhibitors on the incorporation of phosphatidylcholine into *Bacillus* protoplasts

| Addition | Incubation time (min) | % phosphatidylcholine in protoplasts |
|---|---|---|
| None | control[a] | 1.8 ± 1.00 (12) |
| None | 1 | 5.8 ± 1.78 (9) |
| None | 30 | 12.0 ± 0.86 (16) |
| 0.01 M KCN | 1 | 6.0 ± 1.13 (5) |
| 0.01 M KCN | 30 | 12.2 ± 1.25 (5) |
| 0.01 M KF | 1 | 7.7 ± 2.01 (5) |
| 0.01 M KF | 30 | 12.6 ± 1.30 (5) |
| 0.01 M $NaN_3$ | 1 | 8.0 ± 1.21 (5) |
| 0.01 M $NaN_3$ | 30 | 11.1 ± 1.14 (5) |
| Trypsin | control[a] | 2.1 ± 0.85 (4) |
| Trypsin | 1 | 5.9 ± 1.02 (4) |
| Trypsin | 30 | 11.8 ± 0.52 (4) |

[a] Protoplasts not incubated with liposomes

Inhibitors were present during the incubation of protoplasts with liposomes, while trypsin was added after removing the vesicles. Values are means ± SE with the number of experiments in parentheses

bound phosphatidylcholine in protoplasts pre-incubated with liposomes.

*Bacillus* protoplasts were incubated with liposomes containing trapped 6-carboxyfluorescein. The fluorescence yield of this dye increases considerably upon dilution, thus it can be used to detect any vesicle leakiness. Suspensions of protoplasts and 6-carboxyfluorescein-containing liposomes were centrifuged (125000 × *g*, 15 min) to sediment the protoplasts, because of the large amount of light scattering produced by these particles. The fluorescence of the supernatants (fig.1c) is not higher than that of the non-incubated liposome suspension, but lower, forming as a function of time a mirror-image of phosphatidylcholine incorporation. These results suggest that vesicle contents are not spilled out upon interaction with bacterial protoplasts. The increase in fluorescence between 30 and 60 min is probably due to passive leakage of 6-carboxyfluorescein across the protoplast or liposome bilayers.

Finally, protoplasts were incubated with liposomes containing trapped 0.2% phosphotungstate. After 30 min incubation, protoplasts were washed with buffer L and examined under a Philips EM300 electron microscope, at 80 kV, without any additional staining. A typical image is shown in fig.2a; it can be seen that the stain is now inside the protoplast. Most protoplasts appeared stained in the same way. A control protoplast, routinely stained with 0.2% phosphotungstate, is shown in fig.2b; the image obtained with an externally added stain is clearly different from the former. The small, round-shaped images that are seen throughout the field in both preparations may correspond to re-sealed protoplast fragments.

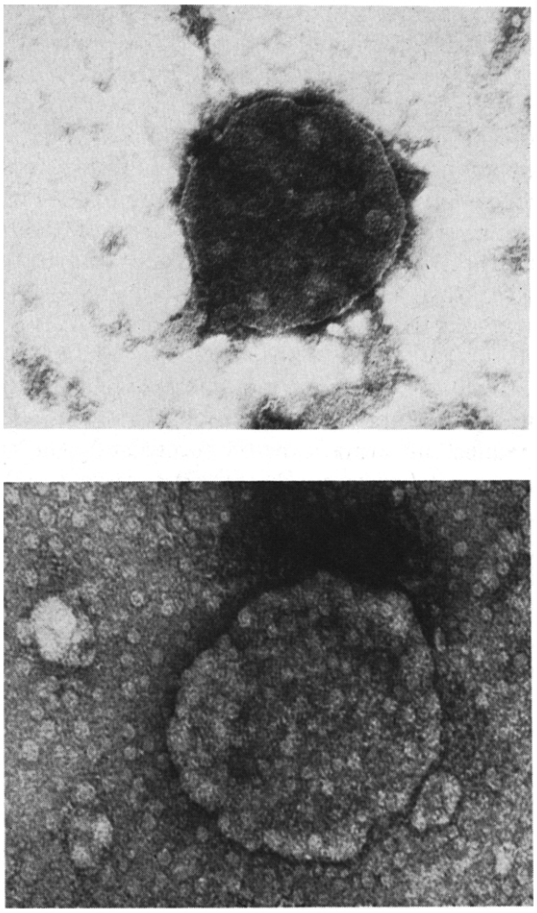

Fig.2. (a) Electron micrograph of a *Bacillus* protoplast. The protoplast preparation was incubated for 30 min with liposomes containing 0.2% phosphotungstate. No external stain was added; see text for details (42000 ×). (b) Electron micrograph of a negatively stained, untreated *Bacillus* protoplast (42000 ×).

## 4. DISCUSSION

Our results show that, when *Bacillus* protoplasts are incubated with sonicated phosphatidylcholine liposomes, the phospholipid appears to be incorporated into the protoplasts. The possibility of protoplast-liposome binding by electrostatic forces is excluded, because of repeated washing with high ionic strength buffer (buffer L, see section 2) after incubating the vesicles with protoplasts. Binding through other (e.g., hydrophobic) non-specific forces may be ruled out after the experiments shown in fig.2. Authors in [2] have distinguished 4 main mechanisms for in vitro liposome–cell interactions, namely stable adsorption, endocytosis, fusion and lipid transfer. They have suggested experimental techniques for distinguishing between these 4 mechanisms. Our results on protoplast–liposome interactions may be rationalized in the terms proposed in [2].

Stable adsorption is the association of intact liposomes with the cell surface, without their internalization. Stable adsorption of phosphatidylcholine (below their $T_c$) to lymphocytes [11], fibroblasts [10] and cultured mouse cells [13] has been demonstrated. Adsorbed vesicles can be released by treatment with trypsin, but not by repeated washing [2,12]. In our case, trypsin failed to reduce the proportion of phosphatidylcholine bound to protoplasts (table 1). Also, the results in fig.1b show a tendency of the lipid P/protein ratio to regain its native value after 60 min incubation. This could be interpreted as the result of an increase in phospholipid catabolism, in order to maintain the appropriate lipid/protein ratio in the membranes; the existence of this regulatory mechanism would support some kind of phospholipid internalization, which does not occur in stable adsorption.

Endocytosis [14] could also be responsible for liposome uptake by protoplasts. This mechanism is markedly reduced in the presence of metabolic inhibitors [2]. Authors in [15] have found that the uptake of sonicated phosphatidylcholine liposomes by fibroblasts is decreased by glycolytic and respiratory inhibitors. However, in our system, none of these inhibitors was effective in reducing the incorporation of phosphatidylcholine into *Bacillus* protoplasts (table 1). In any case, endocytosis is more commonly found with large multilamellar liposomes than with sonicated vesicles [16,17].

Fusion is the merging of the lipid bilayer with the plasma membrane, and concomitant release of liposome contents into the cytoplasmic space, while in lipid transfer there is no cell association with aqueous liposome contents [2]. Therefore, the main experimental test between these two techniques consists of the study of liberation of vesicles contents. Liposomes containing 6-carboxyfluorescein and phosphotungstate are useful in this respect [18]. In our system, there was no sign of dye liberation to the aqueous medium upon interaction with liposomes (fig.1c); in contrast, phosphotungstate was transferred to the inner protoplast compartment, making the protoplasts visible under the electron microscope (fig.2a). These observations are not compatible with a lipid transfer mechanism.

Thus, our experimental results on the interaction of *Bacillus* protoplasts with sonicated phosphatidylcholine vesicles are best described as a cell-liposome fusion process. This kind of fusion was found in similar systems, but using mammalian cells, when liposomes were in the fluid state [15,19]. However, we cannot completely rule out other possible mechanisms; authors in [11] observed only a small inhibition of endocytosis in the presence of metabolic inhibitors. Also, other concomitant processes may be occurring in addition to cell-liposome fusion, such as liposome-mediated protoplast-protoplast fusion [2].

## ACKNOWLEDGEMENTS

This work was supported in part by a grant from CAICYT. The authors are grateful to Dr J.B. Dominguez and Dr A. Alonso for helpful discussion, and to Miss C. Otamendi for the photography.

## REFERENCES

[1] Gratzl, M., Schudt, C., Ekerdt, R. and Dahl, G. (1980) in: Membrane Structure and Function (Bittar, E.E. ed.) pp.59–92, Wiley, New York.
[2] Pagano, R.E. and Weinstein, J.N. (1978) Annu. Rev. Biophys. Bioeng. 7, 435–468.
[3] Poste, G. and Nicolson, G. (1978) Membrane Fusion, Cell Surface Reviews, vol.5, Elsevier, Amsterdam, New York.

[4] Setlow, J.K. and Hollaender, A. (1979) Genetic Engineering: Principles and Methods, Plenum, New York.
[5] Weibull, C. (1953) J. Bacteriol. 66, 688–695.
[6] Singleton, N.S., Gray, M.S., Brown, M.L. and White, J.C. (1965) J. Am. Oil Chem. Soc. 92, 52–53.
[7] Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[8] Bartlett, G.R. (1959) J. Biol. Chem. 234, 446–468.
[9] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[10] Sáez, R., Alonso, A., Villena, A. and Goñi, F.M. (1982) FEBS Lett. 137, 323–326.
[11] Huang, L., Ozato, K. and Pagano, R.E. (1978) Membrane Biochem. 1, 1–26.
[12] Pegano, R.E. and Takeichi, M. (1977) J. Cell Biol. 74, 531–546.
[13] Szoka, F., Jacobson, K., Derzko, Z. and Papadjopoulos, D. (1980) Biochim. Biophys. Acta 600, 1–18.
[14] Silverstein, S.C., Steinman, R.M. and Cohn, Z.A. (1977) Annu. Rev. Biochem. 46, 669–722.
[15] Poste, G. and Papahadjopoulos, D. (1976) Proc. Natl. Acad. Sci. USA 73, 1603–1607.
[16] Gregoriadis, G. and Buckland, R.A. (1974) FEBS Lett. 45, 71–74.
[17] Weissman, G., Bloomgarden, D., Kaplan, R., Cohen, C., Hoffstein, S., Collins, T., Gotlieb, A. and Nagle, D. (1975) Proc. Natl. Acad. Sci. USA 72, 88–92.
[18] Weinstein, J.N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W.A. (1977) Science 195, 489–491.
[19] Papahadjopoulos, D., Poste, G. and Mayhew, E. (1974) Biochim. Biophys. Acta 363, 404–416.
[20] Martin, F.J. and MacDonald, R.C. (1976) J. Cell Biol. 70, 506–514.