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FORMATION OF INSIDE-OUT VESICLES OF *BACILLUS LICHENIFORMIS*

DEPENDENCE ON BUFFER COMPOSITION AND LYSIS PROCEDURE

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Summary

1. The extent to which the cytoplasmic membrane of the Gram-positive bacterium *Bacillus licheniformis* formed inside-out vesicles was studied with the freeze-fracture technique. The membrane orientation appeared to be dependent on the buffer composition as well as on the lysis procedure used.

2. By manipulating these conditions, membrane preparations were obtained with the percentage of inside-out vesicles varying from 15 to 80%.

3. More vesicles had the opposite orientation when the cells were lysed in potassium phosphate buffer than when they were lysed in sodium phosphate buffer. Tris-HCl buffer favoured the formation of inside-out vesicles more than phosphate buffer.

4. Lysis of protoplasts in hypotonic buffers resulted in more inside-out vesicles than did direct lysis of cells in hypotonic media.

5. In an attempt to explain the observed differences, experiments were performed in which the morphology of thin-sectioned lysing cells in sodium phosphate buffer was compared with that in potassium phosphate buffer. The results from these experiments indicate that the formation of inside-out vesicles is brought about by an effect on the membrane itself rather than on the cell wall, on the cell wall membrane association, or on the cytoplasm.

Introduction

Bacterial membrane vesicles are often used in transport and localization studies. These vesicles are formed by fragmentation of the cytoplasmic mem-

brane during or after lysis of bacteria. A certain amount of the membrane vesicles are found to have an orientation opposite to that of the original membrane in the cell, i.e. the inside has become outside and vice versa. Originally this was shown by Steck et al. for erythrocytes [1].

Dependent on the organism used, on the method of vesicle preparation, and on the method of determining the percentage of inside-out vesicles, large differences have been found in this percentage. For example, values have been reported of 0—50% inside-out [2—4] for vesicles derived from the cytoplasmic membrane of *Escherichia coli* by osmotic lysis of spheroplasts. Hare et al. [2], using agglutination with anti-ATPase as a tool, found 50% inside-out and Futai [3], using inhibition of ATPase by antiserum and accessibility of enzymes to substrates, reported 40—60% to be inside-out. On the other hand, Altendorf and Staehelin [4], using the freeze-fracture technique, found that nearly all of the vesicles had the same orientation as in the cell, i.e. they were right-side-out.

Of the methods to determine membrane orientation, the freeze-fracture technique is probably the most reliable. Freeze-fracturing splits biological membranes along a central, hydrophobic plane [5—7], resulting in convex and concave fracture faces, which represent the inner half and the outer half of the membrane, respectively. In bacteria [4,7—9] as well as in most eukaryotic cells [1,5,10], there is an asymmetric distribution of particles on these fracture faces. More of these so-called intramembrane particles appear to be associated with the convex than with the concave fracture face of the cytoplasmic membrane [1,4—10]. This asymmetry can be used to clarify the orientation of membrane vesicles derived from cytoplasmic membranes [1,4,9].

As we are involved in localization studies of the membrane-bound respiratory nitrate reductase of *Bacillus licheniformis*, it was necessary to know what proportion of membrane vesicles was inside-out and which factors influence their formation. This information is needed to assess whether the enzyme is localized on the inside or on the outside of the membrane. Using the freeze-fracture technique we found that, by varying the buffer composition and the lysis procedure, preparations were obtained with the percentage of inside-out vesicles varying from 15 to 80%. Our observations suggest that the effect of buffer composition and lysis procedure on the formation of inside-out vesicles represents a direct effect on the membrane and that it is not affected very much by the cell wall-membrane association or by the underlying cytoplasm.

Materials and Methods

Growth of B. licheniformis. B. licheniformis (Strain S 244) was grown anaerobically at 37°C till the mid-log phase on a medium containing (per l): 25 g Brain Heart Infusion (Oxoid), 2.5 g Bacteriological Peptone (Oxoid), 7.5 g Na_2HPO_4, 4.5 g KH_2PO_4, 3.5 g KNO_3, 50 mg $MgSO_4$, 5 mg $FeSO_4$ and 5 mg $(NH_4)_6Mo_7O_{24}$.

Preparation of vesicles. The cells were either lysed directly or first converted to protoplasts, which were then lysed osmotically.

Direct lysis of cells was done as follows. Cells were washed twice and resuspended at a cell density corresponding to 1.6 g cells (wet weight)/100 ml in 50—200 mM sodium, potassium or sodium/potassium phosphate buffer

(pH 7.0), or in 60 mM Tris-HCl buffer (pH 8.0) with or without 50 mM KCl or NaCl. After addition of lysozyme (Sigma Chem. Co., St. Louis, MO, U.S.A., 0.2 mg/ml), the suspension became clear in about 10 min at 20—22°C.

The buffer used for formation of protoplasts was 65 mM potassium or sodium phosphate buffer, pH 7.0, containing 0.7 M sucrose. After being washed twice in one of these buffers the cells were resuspended therein at the same cell density as above. Lysozyme was added to a final concentration of 0.2 mg/ml and the suspension was incubated at 20—22°C until nearly all of the cells had become spherical. This was usually the case within 45 min as judged by phase-contrast microscopy. The protoplasts were then spun down at 15 000 × *g* for 20 min. They were lysed osmotically; either immediately by homogenization in the appropriate phosphate buffer without sucrose, or slowly by dialysis against buffer without sucrose.

Addition of deoxyribonuclease (Sigma Chem. Co., about 10 μg/ml) to the viscous suspensions and centrifugation for 1 h at 100 000 × *g* and 4°C yielded pelleted membrane vesicles.

Electron microscopy. For freeze-fracturing, the membrane vesicles were washed once with 65 mm potassium phosphate buffer, pH 7.0, supplemented with 30% (v/v) glycerol. They were resuspended in this buffer and frozen in Freon 22 cooled to −150°C with liquid nitrogen. Freeze-fracturing and shadow-casting was performed with a Balzer's apparatus (Balzers A.G., Liechtenstein) at −100°C. The replicas were cleaned with commercial bleach and examined with a Philips EM 300 electron microscope operated at 80 kV. Observation of the replicas made it clear that essentially all the fracture faces fall in one of two categories: one densely covered with particles and one with much less particles. The ratio of the number of these particles on the two membrane faces is about 20 : 1. This high ratio makes it very easy to distinguish the two fracture faces visually. The same ratio applies to convex and concave fracture faces of the cytoplasmic membrane of intact bacteria. It was therefore concluded that convex fracture faces of vesicles with few particles and concave fracture faces with many particles belong to inside-out vesicles. The number of right-side-out and inside-out vesicles was counted and the percentage of inside-out vesicles was computed from these figures.

For thin-sectioning, the material was fixed for 30 min with 0.1% OsO_4, pelleted, and resuspended in 2% agar. The agar was cut into blocks of about 1 mm^3 that were treated for 30 min with 1% OsO_4 in acetate/veronal buffer, pH 6.0, and for 30 min with 1% uranyl acetate in distilled water. The blocks were then dehydrated in acetone and embedded in Vestopal W according to Ryter and Kellenberger [11]. Sections were cut with glass knives on an LKB ultratome III and they were stained with uranyl acetate according to Mollenhauer [12].

Results

Factors affecting inside-out vesicle formation

In a first attempt we investigated whether growth temperature and age of the bacterial culture influenced the orientation of the membrane vesicles after lysis. In this experiment, *B. licheniformis* was grown in batches at 30°C and at 37°C.

After being washed with 65 mM sodium/potassium phosphate buffer (80 mM Na^+, 20 mM K^+, pH 7.0), the cells were either lysed immediately by addition of lysozyme, or aged for 16 h at 4°C and lysed thereafter in the same manner. Freeze-fracturing of the four vesicle preparations obtained in this way showed that they all contained about 20% inside-out vesicles. We may thus conclude that, within the range investigated, growth temperature and age of the bacteria do not influence the orientation of the membrane vesicles.

Other parameters, however, did affect the orientation of the vesicles; especially the buffer composition turned out to be important. Table I shows that vesicles prepared in potassium phosphate buffer contained more inverted vesicles than did vesicles prepared in sodium phosphate buffer or in buffer containing sodium and potassium. The data shown in Table I and the comparison between sodium/potassium phosphate buffers with different Na^+/K^+ ratios show that this ratio is an important parameter for the amount of inside-out vesicles formed from the cytoplasmic membrane of *B. licheniformis*. Furthermore, there appears to be an increase in inside-out vesicles with increasing potassium phosphate concentration up to 75 mM and a decrease at still higher concentrations (Table I).

The Na^+/K^+ effect was also observed with 60 mM Tris-HCl buffer, pH 8.0. The percentage of inside-out vesicles after lysis in Tris buffer containing 50 mM KCl was higher than that in Tris buffer with 50 mM NaCl (67 vs. 43%). Lysis in Tris buffer without NaCl and KCl also resulted in a high percentage of inside-out vesicles (59%) indicating that the Na^+/K^+ balance is not the only factor involved.

In the above experiments, cells were lysed directly in a hypotonic medium during incubation with lysozyme (see Fig. 1, left part). In another series of experiments, the bacteria were first converted to protoplasts, which were then lysed osmotically (see Fig. 1, right part). Table II shows that this way of lysis

TABLE I

PERCENTAGES OF INSIDE-OUT VESICLES AFTER LYSIS OF *B LICHENIFORMIS* CELLS IN PHOSPHATE BUFFERS

B. licheniformis cells (1.6 g wet weight/100 ml) were lysed by lysozyme (0.2 mg/ml) in sodium, potassium, or sodium/potassium phosphate buffer of different molarities (pH 7.0). After lysis, DNAase was added (10 μg/ml) and the membrane fragments were pelleted at 100 000 × *g* for 1 h. They were washed once with 65 mM potassium phosphate, pH 7.0, 30% glycerol, suspended in this buffer, and processed for electronmicroscopical analysis. Figures in parenthesis denote number of fracture faces counted.

Phosphate concentration (mM)	$[Na^+]$ (mM)	$[K^+]$ (mM)	Percent inside-out vesicles
5	—	8	40 (420)
50	—	80	49 (1445)
65	—	100	50 (533)
75	—	120	59 (339)
100	—	160	47 (1202)
150	—	240	40 (324)
200	—	320	27 (513)
65	80	20	23 (1450)
65	20	80	44 (414)
65	100	—	16 (172)

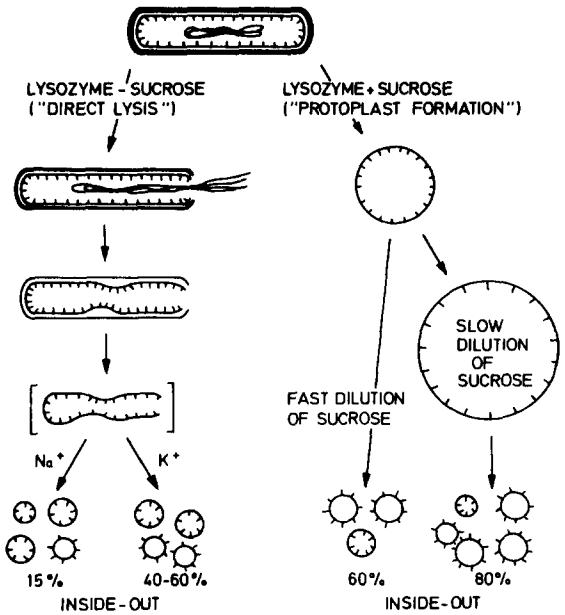

Fig. 1. A schematic representation of the events occurring during the two methods of lysis described in this paper.

always resulted in a high percentage of inside-out vesicles. Especially when the sucrose was slowly diluted by dialysis against buffer without sucrose, many vesicles became inverted. The latter phenomenon might be due to swelling of the protoplasts before they burst. (Corner and Marquis [13] showed that protoplasts of *Bacillus megaterium* became greatly swollen when the stabilizing solute was slowly diluted.) The buffer composition had not much influence on the percentage of inside-out vesicles in case of osmotic lysis of protoplasts although some effect of Na^+ and K^+ was still apparent (Table II).

It is known [14] that divalent metal ions (e.g. Mg^{2+}) stabilize biomembranes whereas the chelating agent EDTA destabilizes them. We, therefore, expected

TABLE II

PERCENTAGE OF INSIDE-OUT VESICLES AFTER OSMOTIC LYSIS OF *B LICHENIFORMIS* PROTOPLASTS

B. licheniformis cells were converted to protoplasts by lysozyme (0.2 mg/ml) in the presence of 0.7 M sucrose in potassium or sodium phosphate buffer (65 mM, pH 7.0). Protoplasts were lysed osmotically either by being homogenized in the appropriate phosphate buffer without sucrose (osmotic shock) or by being dialyzed against phosphate buffer without sucrose (dialysis). For further details, see Table I.

Buffer	Lysis method	% inside-out vesicle
Potassium phosphate	osmotic shock	64 (625)
Sodium phosphate	osmotic shock	60 (616)
Potassium phosphate + 20 mM $MgCl_2$	osmotic shock	63 (551)
Potassium phosphate + 5 mM EDTA	osmotic shock	61 (743)
Potassium phosphate	dialysis	80 (668)
Sodium phosphate	dialysis	66 (660)

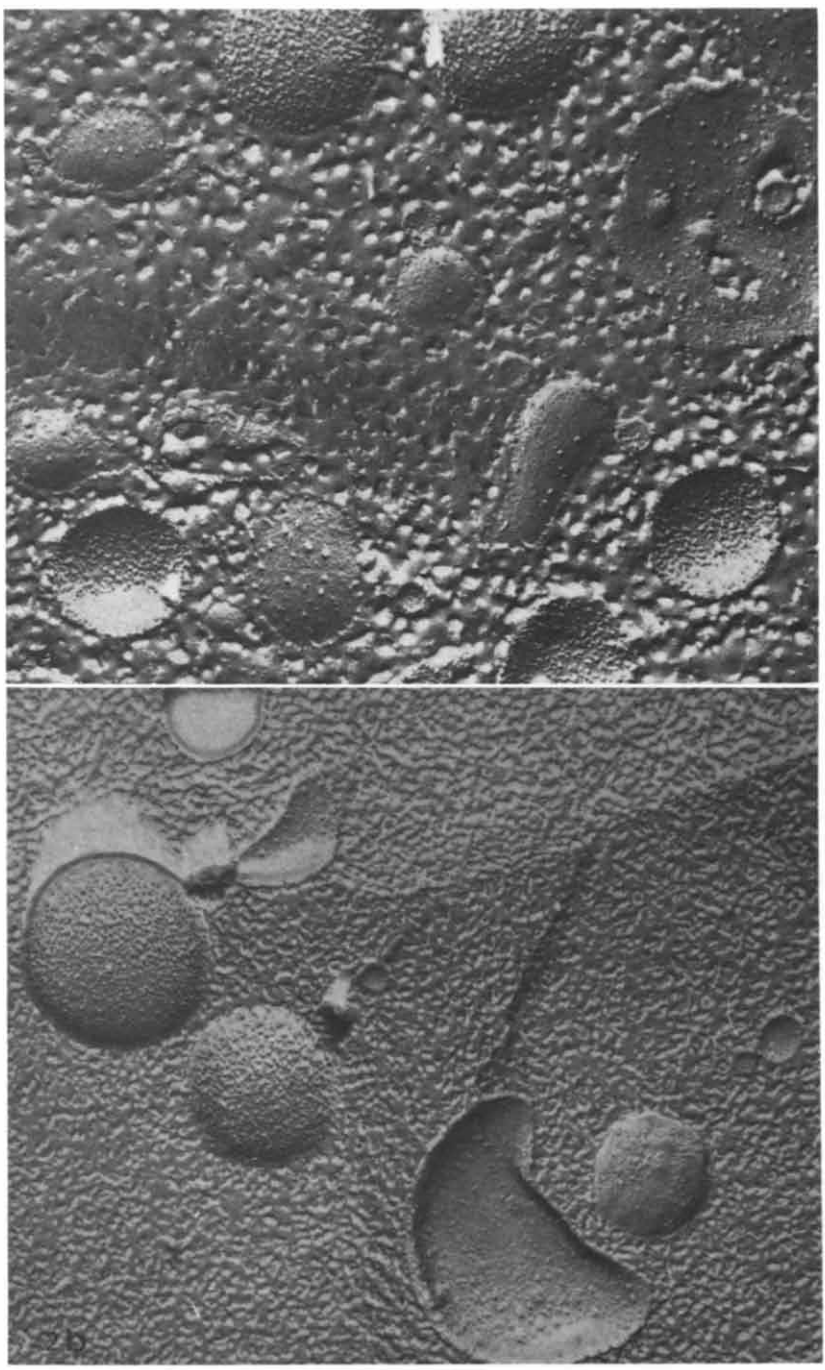

Fig. 2. Freeze-fracture replicas of vesicles preparations. Shadow direction from bottom to top. (a) Vesicles derived from protoplasts osmotically lysed by fragmentation with a homogenizer without sucrose (64% inside-out). (b) Vesicles derived from cells directly lysed in sodium phosphate buffer (16% inside-out). Magnification, ×72 000.

a decrease or increase in the amount of inside-out vesicles by addition to the lysis buffer of $MgCl_2$ or EDTA, respectively. Table II shows, however, that these compounds had no effect on the composition of the vesicle preparations with respect to inside-out percentages.

Fig. 2 illustrates the above-mentioned observations by showing two replicas of vesicle preparations obtained in different ways and containing different amounts of inside-out vesicles. It can be seen that inverted vesicles are usually smaller than right-side-out ones (see also Ref. 4). This means that the percentages of inside-out vesicles presented in this paper cannot be equated with membrane surface areas of the preparations.

Analysis of the Na^+/K^+ effect

The dependence of the percentage of inside-out vesicles on the Na^+/K^+ balance could be due to several mechanisms. One may consider the following possibilities. (a) An effect on cell wall-plasma membrane interactions. The extent to which the membrane is kept in its original position with respect to the cell wall would depend on the tightness of the association between membrane and wall. A lessening of the strength of the association between the two structures would result in greater mobility of the membrane and thus, in more inside-out vesicles. On the other hand, prolonged association during vesicle formation would result in relatively more right-side-out vesicles. (b) An effect on the rate with which the cell wall is degraded by lysozyme. For instance, faster breakdown would result in less cell wall-membrane associations, see (a). (c) An effect on the membrane itself. It is conceivable that binding of Na^+ and/or K^+ to membrane proteins and/or phospholipids renders the membrane more or less rigid. (d) An effect on the cytoplasm. Mincoff and Damadian [15] showed the existence of a cytoplasmic actin-like protein in *E. coli* that polymerizes in the presence of K^+ and that might be bound to the cytoplasmic membrane.

If one or more of these mechanisms had an effect on the orientation that the membrane takes during vesicle formation, differences could be expected to be seen in thin sections between lysing cells that were treated with Na^+ or with K^+. For example, in the presence of Na^+, the plasma membrane would be more frequently associated with the cell wall than in the case of K^+ (possibility (a)). According to possibility (b) a higher percentage of cells would be lysed at given times after lysozyme addition in one case than in the other. The following experiment was therefore performed to test these assumptions. *B. licheniformis* cells were washed twice with 65 mM sodium phosphate buffer (pH 7.0), and with 65 mM potassium phosphate buffer (pH 7.0), respectively. Lysozyme (0.2 mg/ml) was added at zero time and samples were fixed with 0.1% OsO_4 at 0, 1, 2, 3, 5, 7, and 15 min. The samples were then centrifuged for 2 min at 12 000 $\times g$ except for the 15-min samples that were centrifuged for 1 h at 100 000 $\times g$ in order to pellet the membrane vesicles of the completely lysed cells. The pellets were then processed for thin-sectioning as described in Materials and Methods.

Examination of the thin sections revealed no differences between the Na^+- and K^+-treated cells with respect to the rate of lysis. In both preparations 8—10% of the cells were lysed at $t = 0$. After being incubated for 3 min with

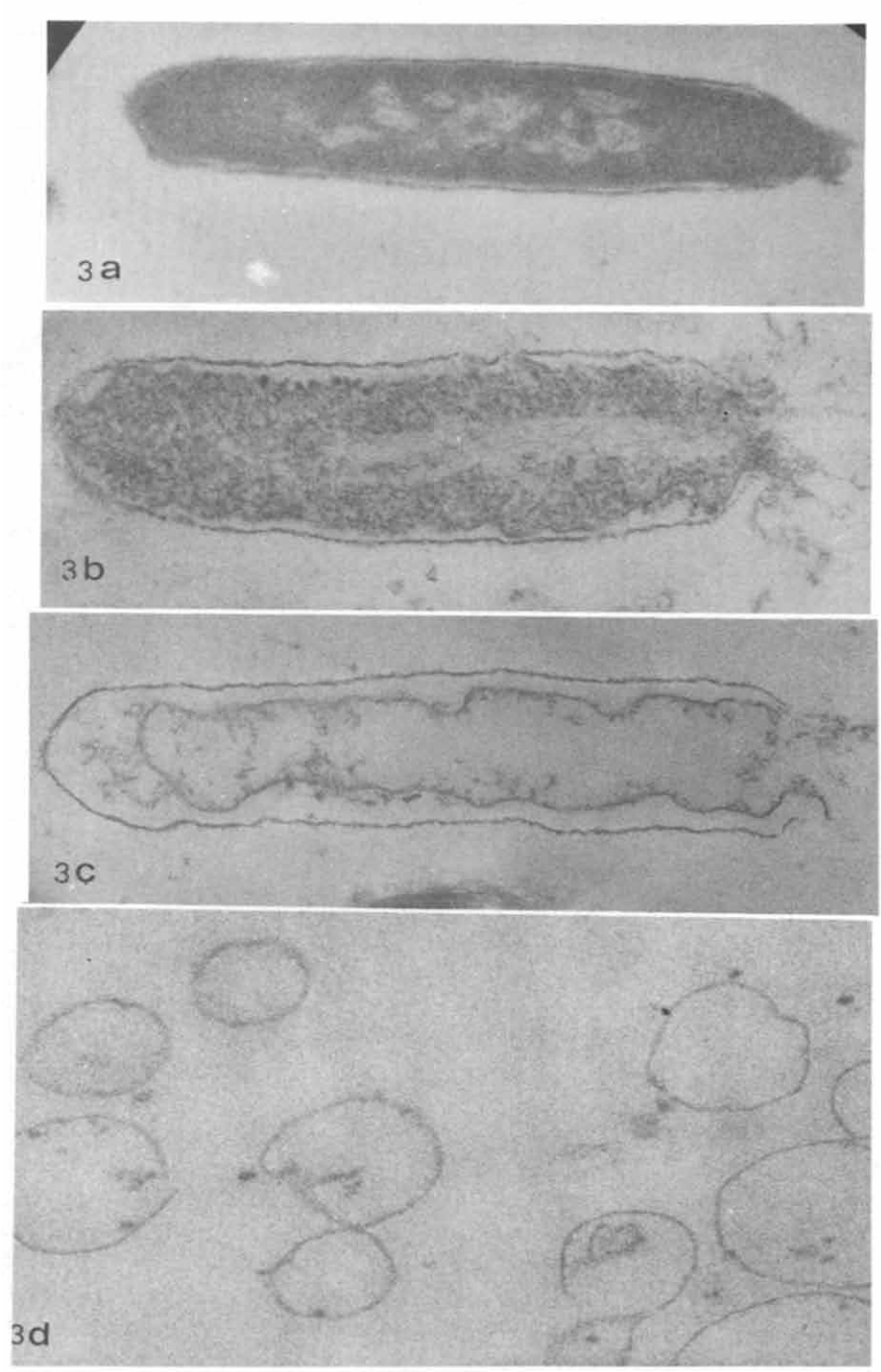

Fig. 3. (a—c) Thin-sections of lysing *B. licheniformis* cells in different stages of lysis (65 mM sodium phosphate buffer, pH 7.0, 0.2 mg lysozyme/ml). (d) After 15 min of lysozyme incubation illustrating membrane vesicles present. Magnification, X42 750.

lysozyme, about 50% of the cells were lysed in both cases and after 7 min lysis was nearly complete. During lysis the cell wall becomes thinner and the cytoplasm is extruded either at one of the poles of a single cell or in the middle of a dividing cell (Fig. 3), i.e. where the cell wall apparently is most sensitive to lysozyme. Fig. 3 also shows that the plasma membrane remains more or less intact and in the original orientation within the remnants of the cell wall. Vesicle formation of the membrane apparently takes place after the breakdown of the peptidoglycan by lysozyme is complete. Apart from the similar behaviour with respect to lysis, the Na^+- and K^+-treated cells appeared identical on an ultrastructural level (not shown). Thus, the Na^+/K^+ effect is presumably not an effect of these alkali ions on the cell wall-membrane association. An effect on the rate of lysis (possibility (b)) is also improbable because Na^+- and K^+-treated cells lysed at an equal rate. Possibility (d) (an effect on a presumed cytoskeleton) is unlikely because formation of vesicles from the cytoplasmic membrane occurs after extrusion of the cytoplasm. Na^+ and K^+ therefore, probably exert their effect on the membrane itself.

Discussion

Some contradictory results occur in the literature concerning the orientation of bacterial membrane vesicles. Most work in this field refers to the cytoplasmic membrane of *E. coli* [2—4,9,16,17]. It seems to us that this inconsistency stems mainly from the use of different methods of determining the inside-out percentage used by the various authors. Hare et al. [2], using agglutination with anti-ATPase and measurement of NADH oxidase activity as a tool, found about 50% of the vesicles to be inside-out. This figure was also reported by Futai [3] who measured binding of anti-ATPase to the membrane vesicles (but apparently did not find agglutination), inhibition of ATPase by the antiserum and accessibility of ferricyanide to the respiratory chain. However, Bisschop et al. [18] showed that ferricyanide does not accept electrons directly from dehydrogenases but via cytochromes and that the reduction of this compound is therefore not a good method for the determination of membrane orientation. Moreover, other authors [16,17] have reported that agglutination studies combined with enzyme activity measurements are not adequate in determining inside-out percentages but lead to the conclusion that membrane vesicles might be mosaics in which some enzymes or parts thereof are translocated from one side of the membrane to the other.

The freeze-fracture technique as used by Altendorf and Staehelin [4] and by Konings et al. [9] is a more reliable method for this purpose because inside-out and right-side-out vesicles can easily be distinguished from each other by the different particle distribution on the fracture faces (see Fig. 2). With this technique we found that inside-out percentages of membrane preparations derived from *B. licheniformis* depend on the buffer composition and on the lysis procedure used; Tris buffer facilitates inside-out formation more than phosphate buffer, K^+ more than Na^+, and osmotic lysis of protoplasts more than direct lysis of cells. These results do not agree with those obtained by Altendorf and Staehelin with *E. coli* [4] and by Konings et al. with *Bacillus subtilis* [9]. The former authors found no difference between buffers containing Na^+ or K^+: in

both cases all vesicles (prepared by the method of Kaback [19]) were right-side-out. However, their vesicles were uniformly very large, the surface area being about the same as that of the membrane of an intact cell. Freezing and thawing resulted in their case in reduction of the average size of the vesicles and in formation of inside-out vesicles, especially when the buffer contained Na^+. Vesicles obtained by us from *B. licheniformis*, on the other hand, were much smaller and must have arisen from fragmentation of the plasma membrane. They were not sensitive to freezing-thawing (results not shown). Konings et al. [9] lysed *B. subtilis* in 0.05 M potassium phosphate buffer, pH 8.0, and found that nearly all vesicles derived from the plasma membrane of this bacterium were right-side-out, only a minority of the enclosed vesicles (15%) having the opposite orientation. Since Altendorf and Staehelin as well as Konings et al. lysed their cells in potassium phosphate buffer (100 mM, pH 6.6, and 50 mM, pH 8.0), the disagreement between their results (all or nearly all vesicles right-side-out) and ours (large amounts of inside-out vesicles depending on the buffer composition and lysis procedure) must have been caused by the different organisms used and/or different conditions of growth, for example, aerobic vs. anaerobic. An indication that this might be so was obtained by Burnell et al. [20]. Unfortunately they did not use the freeze-fracture technique to determine the percentage of inside-out vesicles.

No definite conclusion can be drawn about the ways in which buffer composition and lysis procedure exert their effects on the inside-out percentage of *B. licheniformis* vesicles. However, from the observation that the membrane remains more or less intact within the remnants of the cell wall after lysis and that it apparently fragments at a later stage in the process, it is probable that the buffer composition (for example the Na^+/K^+ balance) exerts its effect on the membrane itself and not on the cytoplasm or on cell wall-plasma membrane association. A possible explanation for this effect is that K^+ causes the membrane to invaginate more than does Na^+. Altendorf and Staehelin [4] have stated that invaginations of the membrane can give rise to inside-out vesicles by a process of pinching off and accumulation within the surrounding protoplast membrane. At a later stage this may disintegrate and set free smaller inside-out vesicles. It may be worthwhile mentioning here that essentially all enclosed vesicles in our preparations were inside-out (see, however, Ref. 13). Recently Lin and Macey [21] showed that in erythrocyte membranes, such invaginations occur and that the extent of invagination is influenced by monovalent and divalent cations, resulting in different amounts of inside-out vesicles. It is conceivable that this process of invagination is enhanced by extension of the membrane which occurs during lysis of protoplasts, especially when the osmotic pressure is slowly lowered by dialysis [7]. In this case, Na^+ and K^+ apparently have less effect on the membrane.

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