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New Procedure for the Isolation of Membrane Vesicles of *Bacillus subtilis* and an Electron Microscopy Study of Their Ultrastructure

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A rapid procedure for the isolation of membrane vesicles of *Bacillus subtilis* is described that minimizes the action of proteolytic enzymes, excreted by this organism, on the membrane proteins. The membrane vesicles obtained have, in addition to a low endogenous respiration rate, a low endogenous activity for transport of amino acids and carboxylic acids. In the presence of the electron donor, ascorbate-phenazine methosulfate, the transport activities for these compounds were comparable to the activities of intact cells. In addition, these activities were retained for a prolonged period of time. Electron microscopy examination of thin sections of the vesicles showed that the preparation consisted almost exclusively of membrane vesicles which were not contaminated with other cell components. The membrane vesicles, which are six to seven times smaller in diameter than protoplasts, often enclosed smaller vesicles. Freeze-etching of intact cells, protoplasts, and membrane vesicles showed that the orientation of the membrane of the vesicles was identical to the orientation of the plasma membrane in intact cells and protoplasts. This also held for the majority of the membranes of the enclosed vesicles, only 15% having the opposite orientation.

A procedure for the preparation of membrane vesicles from gram-positive and gram-negative bacteria has been described by Kaback (6). These membrane vesicles contain almost no cell wall components and are devoid of intracellular constituents (6). They are an ideal tool for the study of integrated membrane functions such as electron transport, phospholipid synthesis, and transport of a wide variety of compounds (7).

The isolation procedure as described by Kaback results in physiologically active membrane vesicles from a wide variety of organisms (6). Some problems arise, however, for organisms that excrete proteolytic enzymes, such as *Bacillus subtilis*. Due to the action of these enzymes, the activity of the membranes obtained is only a fraction of the activity of intact cells. A rapid isolation is required to obtain a reasonably active membrane preparation (12).

In this paper we describe an isolation procedure that circumvents the protoplast formation step and results in the formation of stable, highly active membrane vesicles. The ultrastructure of these vesicles was studied by electron microscopy of thin sections of fixed and embedded vesicles and of freeze-etched vesicles.

MATERIALS AND METHODS

B. subtilis W 23 was grown in a medium containing 0.8% Tryptone (Difco Laboratories, Detroit, Mich.) and 0.5% NaCl at 37 C with vigorous aeration. Cells were harvested at an absorbancy at 660 nm of 1.0 to 1.5, washed with 0.1 M potassium-phosphate, pH 7.3, and centrifuged (20 min at 14,000 × g).

Cells were suspended in 0.1 M potassium-phosphate, pH 8.0, with 20% sucrose at 37 C at a concentration of 1 g (wet weight) per 80 ml. Lysozyme (E. Merck AG, Darmstadt, Germany) was added at a final concentration of 300 µg/ml. The mixture was incubated for 30 min, after which the protoplasts were harvested by centrifugation (30 min at 14,000 × g).

Cells were suspended in 0.05 M potassium-phosphate, pH 8.0, at 37 C at a concentration of 4 g (wet weight) per liter. Lysozyme (E. Merck AG, Darmstadt, Germany), deoxyribonuclease-1 (DNase-1; bovine pancreas DNase, BDH Chemicals Ltd., Poole, England), and ribonuclease (RNase; pancreas, E. Merck AG, Darmstadt, Germany) were added at final concentrations of 300, 10, and 10 µg per ml, respectively. The solution became gradually viscous due to the liberation of deoxyribonucleic acid (DNA). After 15 min of incubation, MgSO₄ was added at a final concentration of 10 mM. Due to the action of RNase and DNase, the viscosity decreased. During this incubation the original turbid cell suspension became

clear (after about 10 min). After 30 min of incubation with $MgSO_4$, sodium-ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 15 mM to facilitate the release of ribonucleic acid (RNA) (6). The incubation was continued for 15 min, after which the $MgSO_4$ concentration was increased to 20 mM. Fifteen minutes later the incubation was stopped by centrifugation of the solution at $20,000 \times g$ for 30 min. The membranes were suspended thoroughly with the Potter-Elvehjem homogenizer, washed twice with 0.1 M potassium-phosphate, pH 6.6, + 10 mM Na-EDTA, and finally resuspended in 0.1 M potassium-phosphate, pH 6.6, at 4 to 6 mg of membrane protein per ml. Portions of 0.5 to 1 ml in thin-walled plastic tubes were rapidly frozen and stored in liquid nitrogen. Protein was determined by the method of Lowry et al. (13).

Transport studies were performed as previously described (6, 11, 12, 15). The filters were dried in counting vials for 20 min at 105 C. After the addition of 10 ml of toluene supplemented with 5 g of 2,5-diphenyloxazol per liter, the samples were counted in a scintillation counter. Uptake experiments with intact cells were performed as described for membrane vesicles.

O_2 utilization was measured with a Clark-type oxygen electrode (YSI, model 53, Yellow Springs Instruments Co., Yellow Springs, Ohio) connected to a Servogor (model RE 511) recorder as described previously (15).

Pellets of intact cells and protoplasts were fixed for 6 h at 4 C in Veronal acetate-buffered (8) 2.5% glutaraldehyde, supplemented with 20% sucrose in the case of protoplasts to prevent lysis, washed for 3 h in several changes of the Veronal acetate buffer, and postfixed for 10 h at room temperature (22 C) in Veronal acetate-buffered 1% OsO_4 . After washing with Veronal acetate buffer, the samples were stained for 30 min in 1% uranylacetate in double distilled water.

Pellets of membrane vesicles were fixed and stained as described for intact cells, except that the periods of fixation and postfixation were reduced to 2.5 h and 1 h, respectively. The samples were dehydrated in a graded series of ethanol prepared by dilution of 96% ethanol with double distilled water containing uranylacetate in a saturated concentration. The material was embedded in Vestopal W. Sections (20 to 50 nm) were cut with a diamond knife on an LKB ultramicrotome, picked up on uncoated 200 mesh grids, and in some cases stained with lead citrate (25) and uranylacetate (10).

After centrifugation cells, protoplasts, and vesicles were quickly frozen in liquid Freon 12 either directly or 20 to 60 min after suspension in a 25% solution of glycerol in Veronal acetate buffer and stored in liquid nitrogen. The specimens were freeze-fractured, etched for 90 s, shadow-casted with platinum-carbon, and replicated with carbon in a Balzers freeze-etching unit (Balzers AG, Liechtenstein) by the method of Moor (17). Replicas were floated off on double distilled water, cleaned with 70% chromic acid, rinsed with distilled water, treated with 20% sodium hydroxide, and finally washed with several changes of double distilled water. The replicas were collected on bare

300 mesh copper grids and dried at room temperature. Specimens were examined in a Philips EM 300 electron microscope at 80 kV.

The orientation of the membranes can be compared with the orientation of the plasma membrane in intact cells and protoplasts by freeze-etching, since, upon freeze-fracturing, mainly two faces (a convex

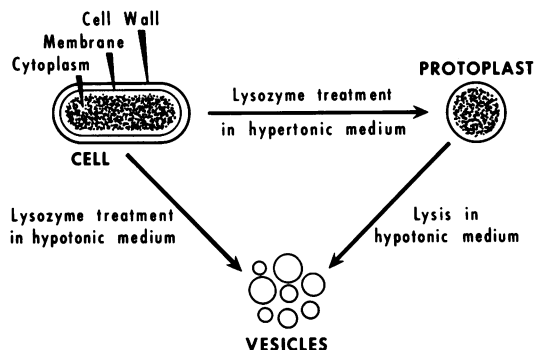


FIG. 1. Scheme of the two isolation procedures discussed in the text.

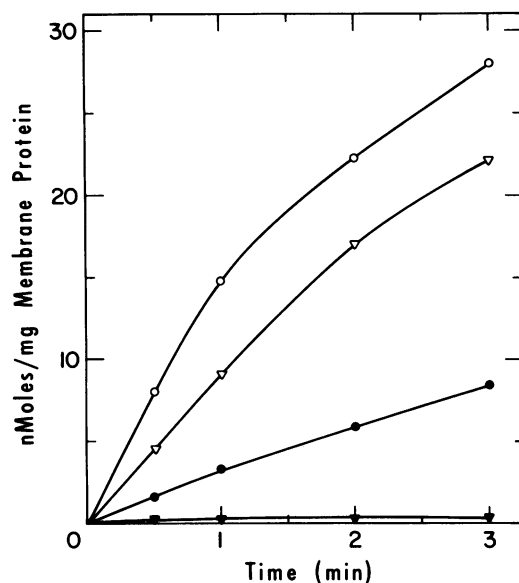


FIG. 2. Comparison of the uptake of *L*-glutamate by intact cells and by membrane vesicles. Symbols: \circ , intact cells; Δ , membrane vesicles in 50 mM imidazole-chloride, pH 6.6, with potassium-ascorbate (10 mM) and phenazine methosulfate (10 μ M); \bullet , membrane vesicles in 50 mM potassium-phosphate with potassium-ascorbate (10 mM) and phenazine methosulfate (10 μ M); ∇ , membrane vesicles in 50 mM imidazole-chloride, pH 6.6, without electron donor. Specific activity of *L*-glutamate (Radiochemical Centre, Amersham, Buckinghamshire, England) was 270 mCi/mmol. Final concentration of *L*-glutamate used was 9.3 μ M. The membrane protein content of whole cells was assumed to be 15% of the total protein (6).

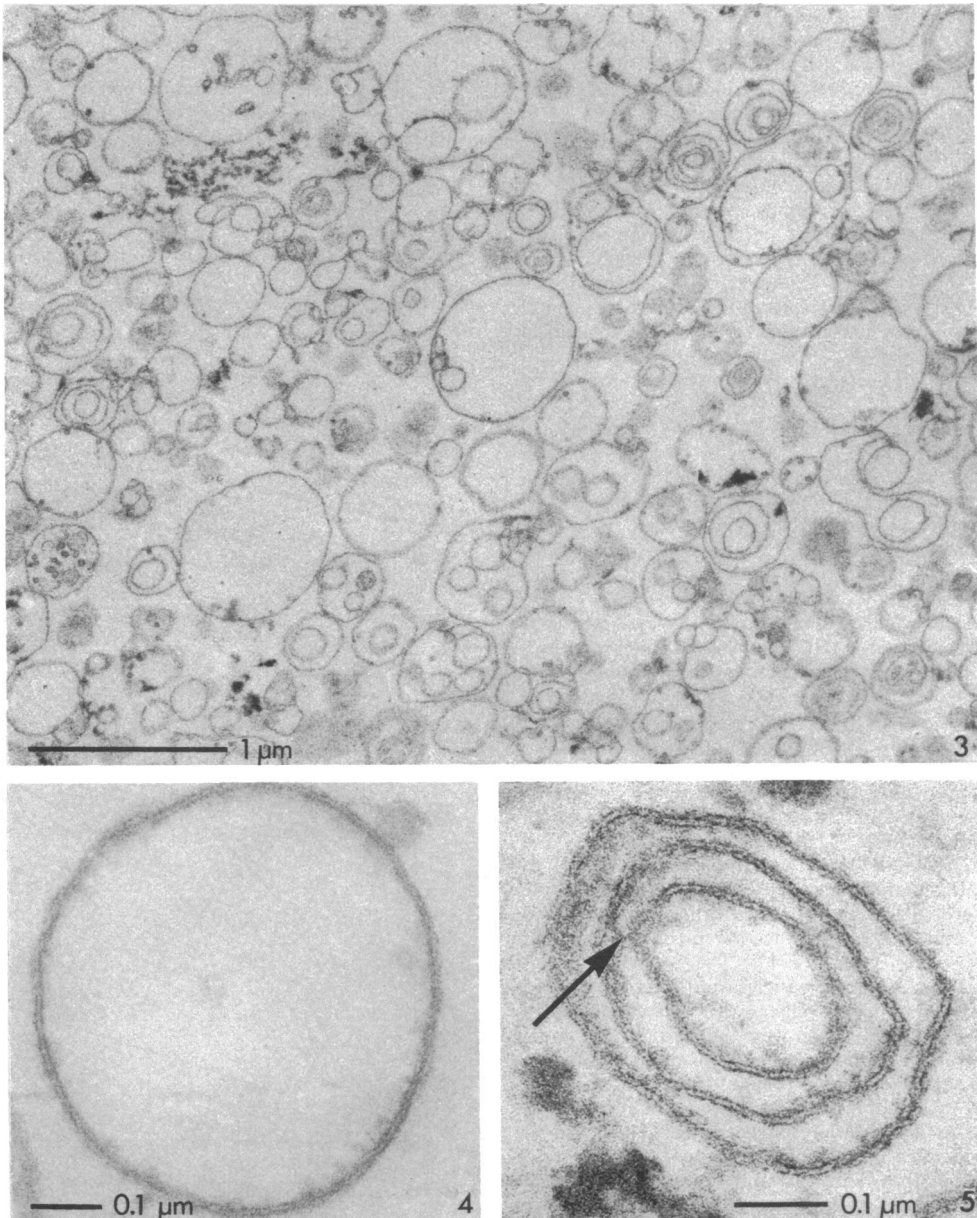


FIG. 3-5. Thin sections of *B. subtilis* W 23 membrane vesicles. Fig. 3, survey of a typical membrane vesicle preparation obtained by the isolation procedure described. Fig. 4, membrane vesicle without internal vesicles. Fig. 5, membrane vesicle with concentrically arranged internal vesicles. Arrow indicates the contact between the internal vesicles.

and a concave face being morphologically different) of the cytoplasmic membrane of bacteria (5, 23, 28, 29, 31, 32) and other organisms (19, 21) have been observed. These convex and concave membrane faces most likely result from cleaving of the membrane along a central plane (1, 21, 28, 29, 33) and are referred to as the inner fracture face (IFF) and the outer fracture face (OFF), respectively.

RESULTS

The isolation procedure for membrane vesicles described by Kaback (6) consists, in essence, of two steps (Fig. 1): the conversion of the microorganism into an osmotically sensitive form (protoplast or spheroplast) by treatment with lysozyme (for gram-negative organisms in

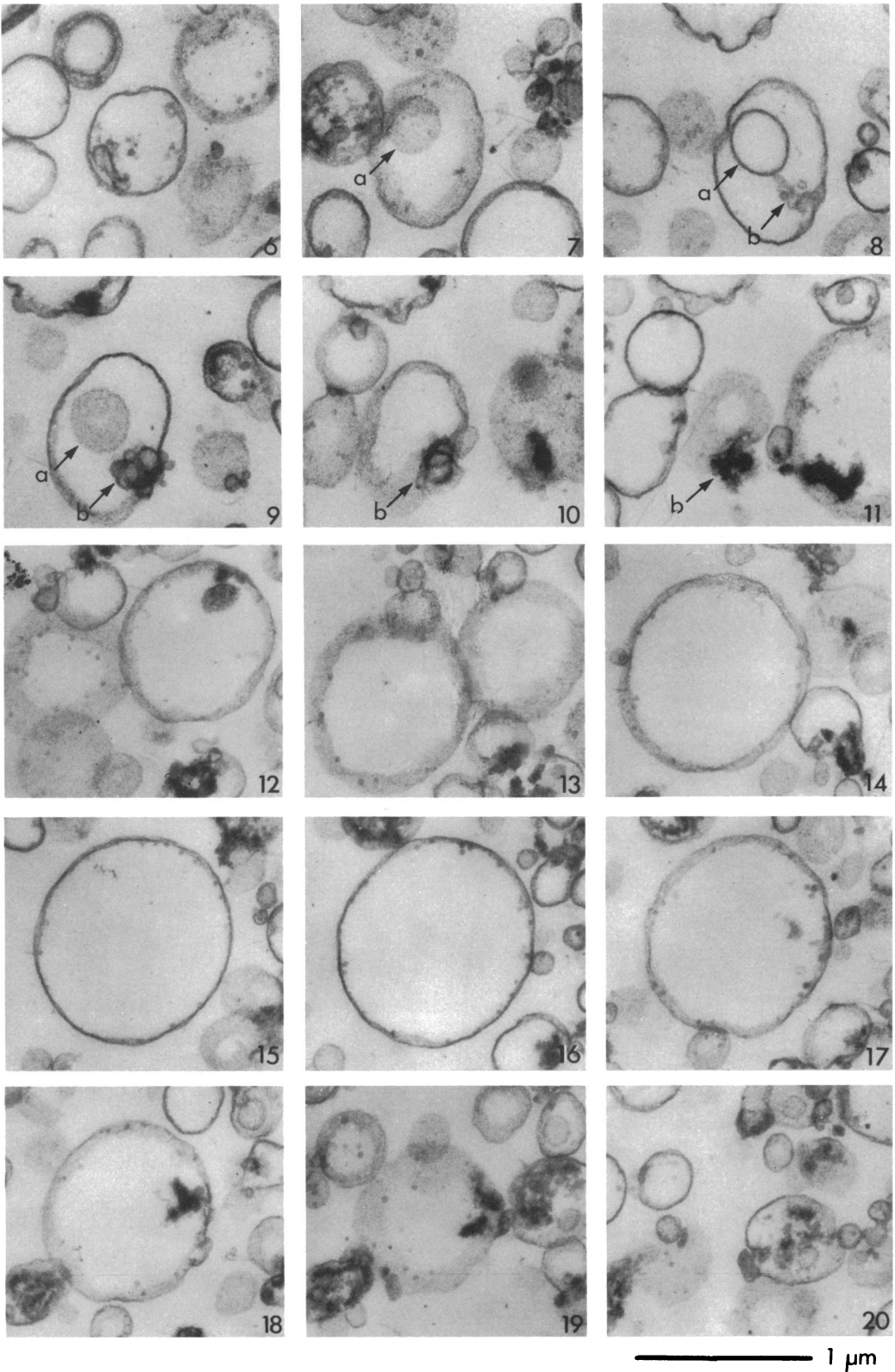


FIG. 6-11. Serial sections of a membrane vesicle with an internal vesicle. The internal vesicle (arrow a) is connected with the outer membrane. A complex of small vesicles connected to the membrane of the outer vesicle is situated partly inside and partly outside this vesicle (arrow b).

FIG. 12-20. Serial sections of a membrane vesicle without internal vesicles.

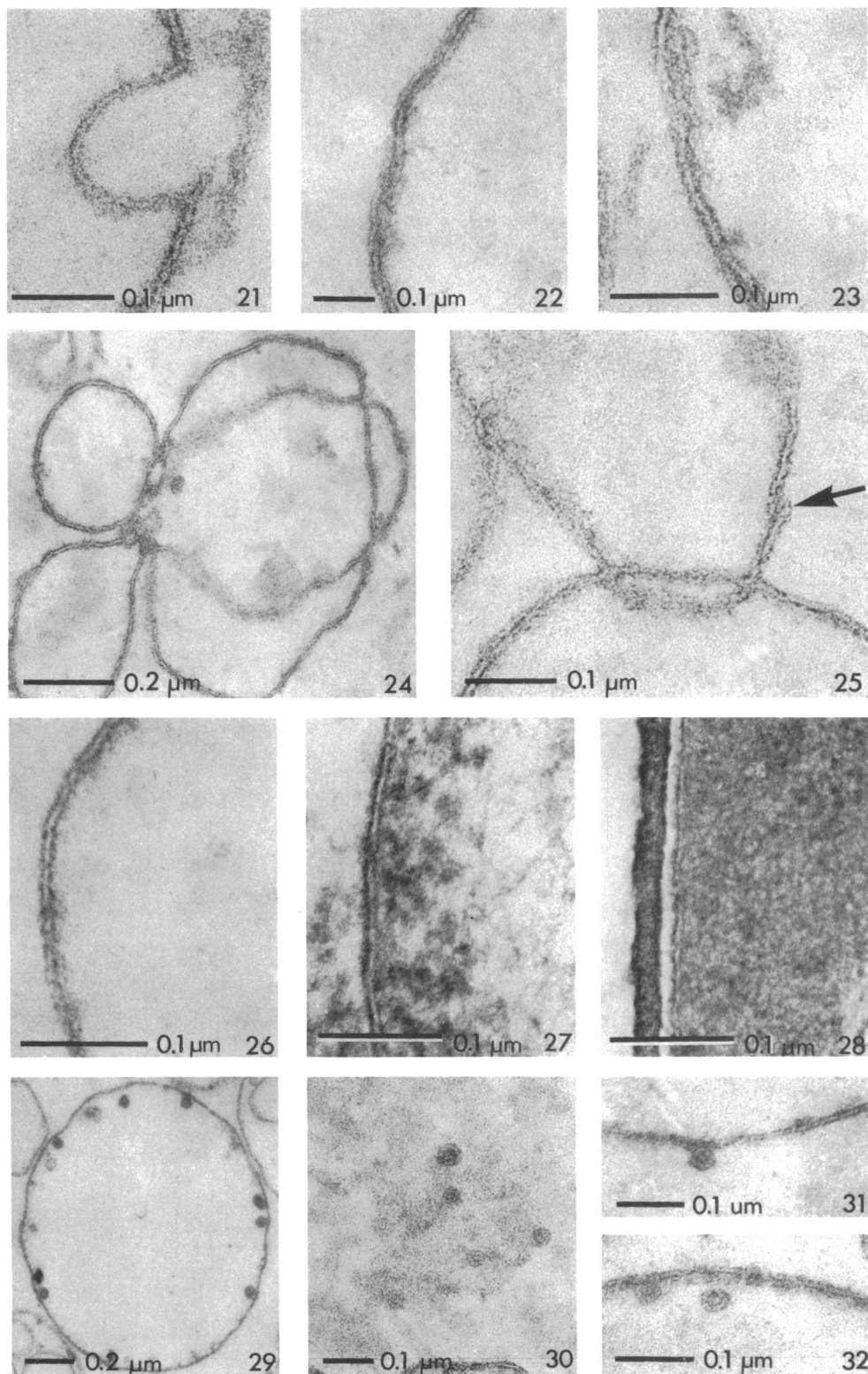


FIG. 21. Detail of a membrane vesicle showing an invagination-like configuration.
FIG. 22. Detail of a vesicle membrane showing three electron-dense layers separated by two layers of low electron density. Note the somewhat twisted-like configuration.
FIG. 23. Detail of a vesicle membrane suggesting the overlap of membrane sheets.
FIG. 24. Complex structure of connected and interlocking vesicles.
FIG. 25. Detail of two interlocked vesicles. Note the continuity of the outer electron-dense layers of the two membranes, with the exception of the interlocking area where the outer dense-layer of the membrane of one

the presence of EDTA) in a hypertonic medium (usually 20% sucrose), followed by a controlled lysis in a hypotonic medium in the presence of nucleases and a chelating agent (e.g., EDTA). However, when this method is applied for organisms like *B. subtilis* that excrete proteolytic enzymes, the isolated membrane vesicles are labile and lose nearly all their activity within a few hours at 0 C (12). The time required for protoplast formation seems to be of particular importance for the activity of the membrane vesicles obtained. During this step proteolytic enzymes are released into the incubation mixture. Figure 1 shows a new isolation procedure, described in detail in Materials and Methods, that circumvents the protoplast formation step. In this procedure the cells are treated with lysozyme in a hypotonic medium (0.05 M potassium-phosphate, pH 8.0) (Fig. 1). This leads to a partial hydrolysis of the cell wall followed by immediate lysis which results in the formation of membrane vesicles. The liberated DNA and RNA is hydrolysed by the DNase and RNase in the incubation mixture, and the remaining cell wall pieces are further hydrolyzed by continued incubation (in total 30 min) with lysozyme. The isolation procedure can be performed in 2 to 3 h, in contrast to the 10 to 12 h required for the previously described isolation procedure.

The transport activity of the membrane vesicles is compared with the activity of intact cells in Fig. 2. In the absence of an electron donor, almost no uptake of L-glutamate is observed, indicating that no endogenous energy source is present in the membrane vesicles. This is supported by the observation that oxygen consumption by the membrane vesicles is less than 2 nmol of oxygen per mg of membrane protein per min. Uptake experiments with membrane vesicles in an incubation mixture containing 50 mM potassium-phosphate, pH 6.6, and ascorbate-phenazine methosulfate as electron donor (11, 12) show an initial rate of L-glutamate transport of about 25% of that of intact cells. The initial rate of L-glutamate uptake in an incubation mixture containing 50 mM imidazole-chloride, pH 6.6, is more than 50% of that of

intact cells (W. N. Konings, manuscript in preparation). As was shown previously (12), L-glutamate is recovered from the membrane vesicles as free L-glutamate. Comparable results are obtained with other amino and carboxylic acids. The membrane vesicle preparation is very stable with regard to the transport activities. Storage of the membrane vesicles for 24 h at 0 C results in a decrease of the transport activity of less than 10%.

The described isolation procedure results in a preparation almost exclusively containing membrane vesicles devoid of cell wall fragments and intracellular components (Fig. 3). The vesicles appear as closed circles or ovals, which frequently enclose one or more often concentrically arranged "internal vesicles" (Fig. 3-20). The internal membranes usually are connected with the surface of the enclosing vesicle (Fig. 6-11). Concentrically arranged internal vesicles often seem to be connected to each other at one point (Fig. 3). The following mechanisms might be responsible for the formation of internal vesicles. (i) Invagination(s) of the surface of a vesicle, as may be indicated by structures shown in Fig. 21. However, these structures were only found incidentally. (ii) Enclosure of membrane vesicles in larger sheets of membranes. However, such a mechanism does not give a satisfactory explanation for the observation that the internal vesicles generally are attached to the surface of the enclosing vesicle. (iii) Interlocking of two or more vesicles, as indicated by frequently observed structures like those shown in Fig. 24 and 25.

The dimensions of the membrane vesicles were determined from serial sections by measuring the maximal diameter of the largest profile of a vesicle in the "longitudinal" direction and in a direction perpendicular to that. The two dimensions distinguished have mean values of $0.31 \pm 0.19 \mu\text{m}$ and $0.24 \pm 0.17 \mu\text{m}$. Since the corresponding diameters of protoplasts are $1.91 \pm 0.18 \mu\text{m}$ and $1.47 \pm 0.17 \mu\text{m}$, it can be concluded that the integrity of the original plasma membrane is lost during the vesicle preparation, resulting in the formation of more than one vesicle per cell.

vesicle seems to be continuous with the inner dense layer of the membranes of the second vesicle. Arrow indicates overlap of membranes.

FIG. 26-28. Details of the membrane of vesicles (Fig. 26) and the plasma membranes of protoplasts (Fig. 27) and intact cells (Fig. 28). Note the symmetry of the membrane of vesicles compared with the different electron density of the outer and inner layers of the plasma membrane in intact and protoplasted cells.

FIG. 29. Survey of a membrane vesicle showing numerous membranous spheres at the inner surface of the vesicle.

FIG. 30. Section containing a sheet of membrane with membranous spheres.

FIG. 31, 32. Membranous spheres located on the outer surface (Fig. 31) and the inner surface (Fig. 32) of a vesicle. Note the unit membrane structure of the spheres.

The morphology of the membranes of vesicles was studied in detail by examination of ultrathin sections (20 nm). It appeared that the triple-layered membranes of vesicles are always symmetric (Fig. 26), in contrast with those of protoplasts and intact cells where the outer surface layer is more electron dense than the inner surface layer (Fig. 27 and 28). At some places membranes of vesicles appear as twisted structures formed by three electron-dense layers which are separated by two layers of low electron density (Fig. 22). Another configuration of membranes (Fig. 23) is suggestive of two overlapping sheets of membranes. Frequently, small membranous spheres (40 to 50 nm in diameter) associated with the inner surface and/or sometimes with the outer surface of the vesicles have been observed (Fig. 29-32).

As has been stated in Materials and Methods, the orientation of the membranes of vesicles can be determined by freeze-etching, since the convex IFF of several strains of bacteria are densely covered with 50- to 150-nm particles, whereas the concave OFF shows a much looser arrangement of similar particles (9, 27, 28, 29, 31).

In intact cells, protoplasts, and membrane vesicles, convex membrane fracture faces densely covered with small particles (Fig. 33, 34, 36 and 38) and concave fracture faces with a lower particle density (Fig. 33, 35, 37 and 39) were found. Although more than a thousand freeze-etched vesicles were examined, no membranes with an opposite orientation were observed. This strongly indicates that the membrane of the vesicles is oriented as the plasma membrane of intact and protoplasted cells. Small spheres (40 to 65 nm) usually connected with the inner surface of the membrane were often observed in protoplasts (Fig. 40) and membrane vesicles (Fig. 43) but not in intact cells. These spheres are, in all probability, identical with the membranous spheres observed in thin sections of vesicles (Fig. 29-32). In freeze-etched vesicles they are found only connected with the outer unit membrane, just as the membranous spheres seen in thin sections. However, the fracture faces of the spheres are always smooth, in contrast to those of the vesicle membranes.

The existence of internal vesicles is also shown by freeze-etching (Fig. 42 and 43). The orientation of the membranes of 500 internal vesicles was studied. In 15% of the internal vesicles, the concave fracture faces instead of the convex fracture faces were densely covered with particles (Fig. 42), which indicates that the orientation of these membranes is the opposite of the orientation of the membrane of the outer

vesicles. The majority (85%) of the internal vesicles, however, have membranes with an orientation similar to that of the enclosing vesicles (Fig. 43).

DISCUSSION

The procedure described in this paper for the isolation of membrane vesicles of *B. subtilis* has several advantages over the previously described procedure (6): the membrane vesicles obtained (i) have transport activities that are in the same order of magnitude as the activities of intact cells; (ii) they retain their transport activities for a prolonged period of time; and (iii) they are isolated much more rapidly. An additional advantage of this isolation procedure is that it permits isolation in a defined medium throughout the whole procedure, which might be of importance for the study of the effects of different ions inside or outside the membrane vesicles. The membrane vesicles do not contain intracellular components, as shown by electron microscopy of thin sections of vesicles and as indicated by the low endogenous activity with regard to transport activity and oxygen consumption.

The average diameter of the membrane vesicles is six to seven times smaller than that of protoplasts. The surface to volume ratio of the membrane vesicles therefore will also be six to seven times smaller than that of protoplasts or intact cells. For *Escherichia coli* an internal volume was calculated of 49 μ liters of membrane protein per mg (3). By using this same value for *B. subtilis*, an internal volume of the membrane vesicles per milligram of membrane protein of 7 to 8 μ liters can be calculated. Taking into account the fact that internal membranes and open membrane structures do not contribute to the total inner volume, this calculated inner volume agrees reasonably well with the experimentally determined inner volume of 2 to 3 μ liters per mg of membrane protein (11).

The freeze-etch electron microscopy studies clearly reveal that the orientation of the membrane of the outer vesicles is right-side out. An opposite orientation of these membranes was never observed. However, such an orientation was observed in some membrane vesicles enclosed in other membrane vesicles, which indicates that these membrane vesicles are the result of invaginations. The majority of the internal vesicles are certainly not the result of invaginations, but might be formed by inclusions in bigger vesicles, possibly during the homogenization procedures employed in the

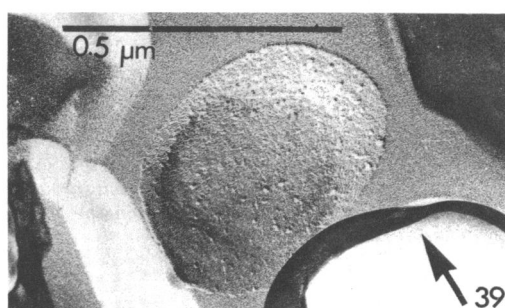
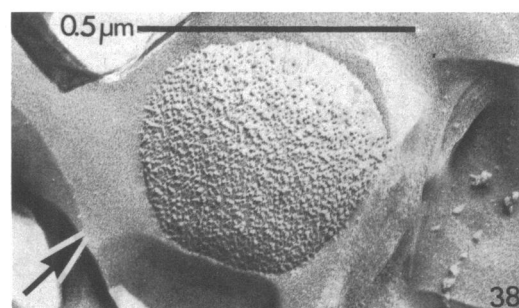
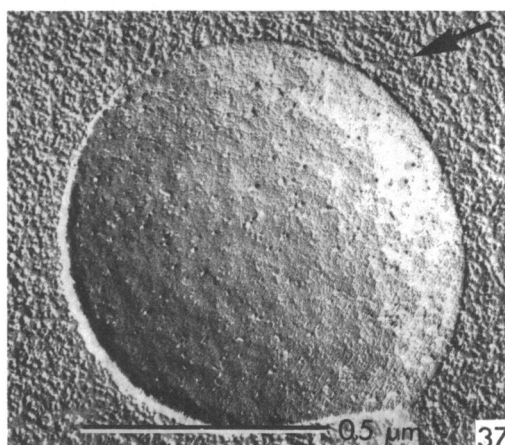
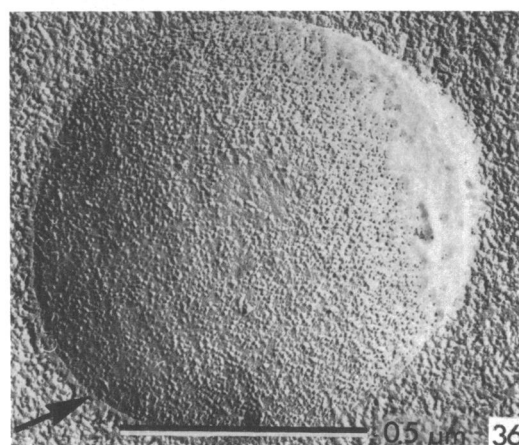
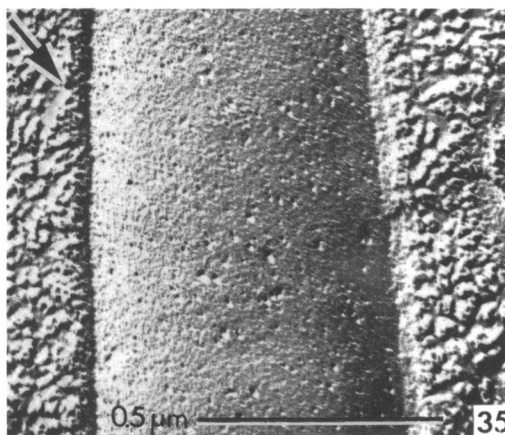
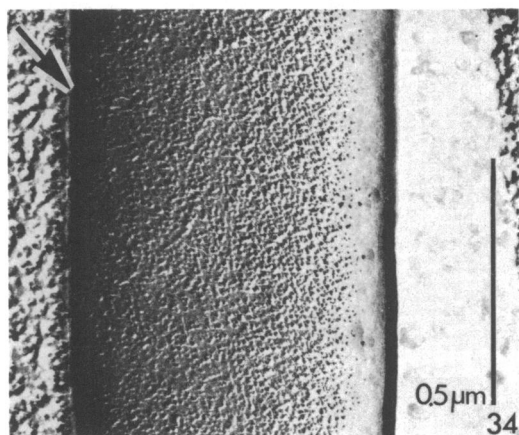
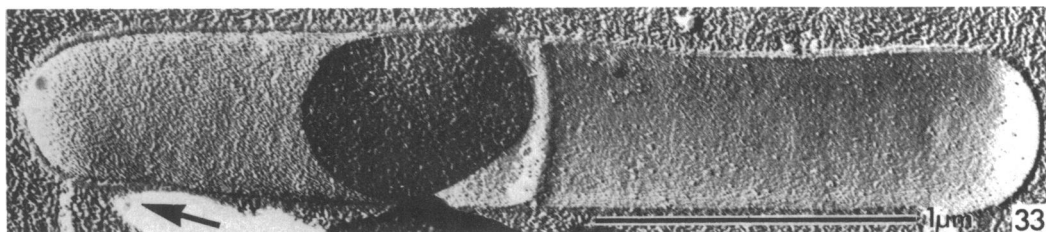


FIG. 33. Replica of a freeze-etched cell of *B. subtilis* W 23 showing two fracture faces of the plasma membrane. Note the high density of particles on the convex face (IFF) and the lower density on the concave fracture face (OFF). The arrow in this and the next figures indicates the direction of shadow.

FIG. 34, 35. Replicas of freeze-etched intact cells showing the IFF (Fig. 34) and the OFF (Fig. 35) of the plasma membrane.

FIG. 36, 37. Replicas of freeze-etched protoplasts showing the IFF (Fig. 36) and the OFF (Fig. 37) of the plasma membrane.

FIG. 38, 39. Replicas of freeze-etched membrane vesicles showing the IFF (Fig. 38) and the OFF (Fig. 39) of the membrane. Note the similarities between, on the one hand, the inner fracture faces and, on the other hand, the outer fracture faces of the vesicle membranes and plasma membranes of protoplasted and intact cells.

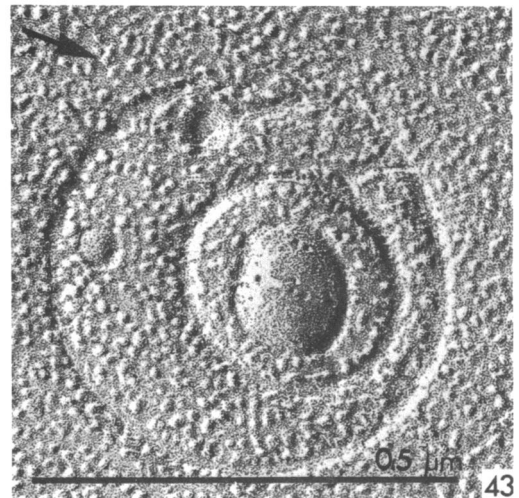
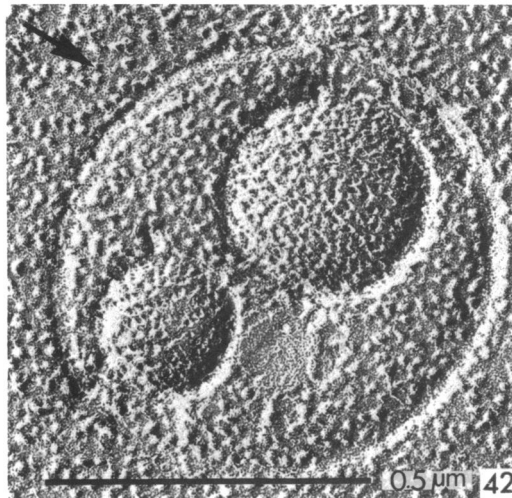
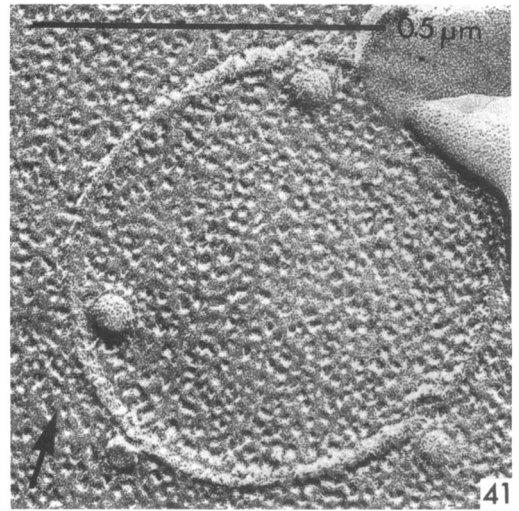
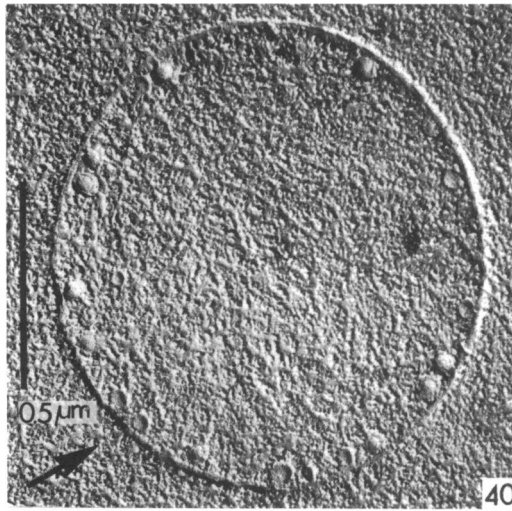


FIG. 40. Replica of a freeze-etched section through a protoplast showing membranous spheres connected with the inner surface of the vesicle. Note the smooth faces of the spheres. The arrow in this and the next figures indicates the direction of shadow.

FIG. 41. Replica of a freeze-etched section through a membrane vesicle showing smooth membranous spheres.

FIG. 42. Replica of a freeze-etched section through a membrane vesicle with internal vesicles. Note that the concave fracture faces of the internal vesicle membranes are densely covered with particles, indicating the outside-in orientation of the membrane of these internal vesicles.

FIG. 43. Replica of a freeze-etched section through a membrane vesicle with concentrically arranged internal vesicles. Note the low particle density on the concave fracture face of the innermost vesicle, indicating its outside-out orientation.

later stages of the isolation process or by interlocking of vesicles. This conclusion agrees with results of Kaback (6) with respect to the orientation of the membranes of vesicles of *E. coli*.

The electron microscopy examination of thin sections indicates that the sealing of the membranes in vesicles may occur via a direct contact

of the edges of the membrane sheets or via an overlap of membrane sheets.

The membranous spheres (40 to 65 nm in diameter) observed in thin sections of membrane vesicles and both in freeze-etched protoplasts and vesicles have smooth fracture faces and are associated with the membrane of the

outer vesicles. It has been suggested that smooth vesicles (60 nm in diameter) observed in other bacilli are related to vesicular mesosomes (22, 23) or are vesicular remnants of mesosomes (26, 30).

The particles exposed by freeze-fracturing of the membranes appeared to be randomly arranged on the convex fracture faces. In some bacteria the arrangement of the particles is dependent on the physiological condition of the cells (4, 31, 32). The particles on the membrane faces have been related to biochemical functions located in plasma membranes (2, 14, 16, 18, 20, 30, 34). The number of particles on the inner fracture faces of vesicles prepared from cells grown on different media varies significantly. A possible correlation between functional properties of the membrane vesicles and the particle density will be investigated.

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