UNIQUENESS AND LOCATION OF THE FRACTURE PLANE
IN THE PLASMA MEMBRANE OF *BACILLUS SUBTILIS*

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The structural features observed in freeze-fractured bacterial membranes have, in the now numerous reports, generally been interpreted as belonging to the external membrane surfaces. However, several lines of evidence indicate the occurrence of membrane splitting as contrasted to cleavage along both surfaces in freeze-fractured biological membranes (for a review see Branton [1969]).

In earlier observations on the plasma membrane of *Bacillus subtilis* we have assumed that fracturing occurred along both surfaces of the membrane (11, 12). The aim of the present investigation on *B. subtilis* has been twofold: (a) to verify whether the concept of a unique fracture plane applies to its plasma membrane; and (b) to attempt to locate the fracture plane in or at the membrane. An answer to the first question has been sought by making complementary freeze-fracture replicas as has been done recently for other membranes (5, 17, 21, 22). The location of the fracture plane has been investigated by making thin sections of freeze-fractured cells (3, 4, 8).

MATERIALS AND METHODS

Culture and Medium

B. subtilis, strain Marburg, was grown aerobically in heart infusion broth (Difco Laboratories, Inc., Detroit, Mich.), on a shaker at about 37°C. Cells were collected in the exponential phase of growth by centrifugation at room temperature for 15 min at 7000 g.

Freeze-Fracturing

Complementary replicas were made with a device similar in design to the one first described by Steere and Moseley (17). It consists of two holders which can be closed on a hinge; the lower holder is attached to the screw cap of the specimen stage of the Balzers freeze-etch unit. The two holders contain complementary holes, and after the specimen suspension is inserted into the holes, the holders are closed on the hinge. Holders and screw cap are dipped in liquid nitrogen and the whole assembly is screwed onto the precooled object stage (−150°C). After the temperature of the object is raised to −100°C *in vacuo*, the holders are unlocked. This is carried out by pushing the microtome arm against a small protruding part of the upper holder. Immediately thereafter the fractured surfaces are replicated according to Moor (9). The replicas are then cleaned and mounted on Formvar-coated copper grids (Belden Mfg. Co., Chicago, Ill., supplied by VECO, Eerbeek, Holland) according to the usual procedure (10). Because of their larger size, yeast cells have been used as markers to find the complementary parts of the fractured bacteria.

Thin Sectioning of Fractured Bacteria

Bacteria contained in agar blocks (16) were fixed in osmium tetroxide and posttreated with uranyl acetate (12, 16). An agar block was placed on a scratched copper disc and, after freezing in liquid Freon 22 (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.), was affixed on the object stage precooled to −150°C. A cut was made with the microtome knife at −100°C. The fractured agar block was then thawed and dehydrated in a graded series of acetone (12, 16). A flat embedding was done in Vestopal W (16). Care was taken to orient the fractured agar blocks in such a way that the fractured surface could be located easily; this was facilitated by giving the agar blocks characteristic shapes. Sections were cut normal to the fractured surface.

Electron Microscopy

Electron micrographs were taken with a Philips EM 200 or EM 300 electron microscope operating at 80 kv. The micrographs of freeze-fractured bacteria are printed in negative in order to produce a "natural" black shadow. The direction of shadowing is indicated by an arrow in the lower right-hand corner of each micrograph.

RESULTS

For *B. subtilis*, no more than two faces of the plasma membrane have been described (11, 15): a convex face densely packed with particles, and

a concave face with far fewer particles. Both faces were found to be nonetchable (cf. references 11 and 13). For convenience we will denote the former as a convex rough face and the latter as a concave smooth face.

Complementary Fracture Faces

The examination of complementary replicas of freeze-fractured *B. subtilis* reveals that a convex rough face is apposed to a concave smooth face and vice versa (Figs. 1 and 2). The shapes of the
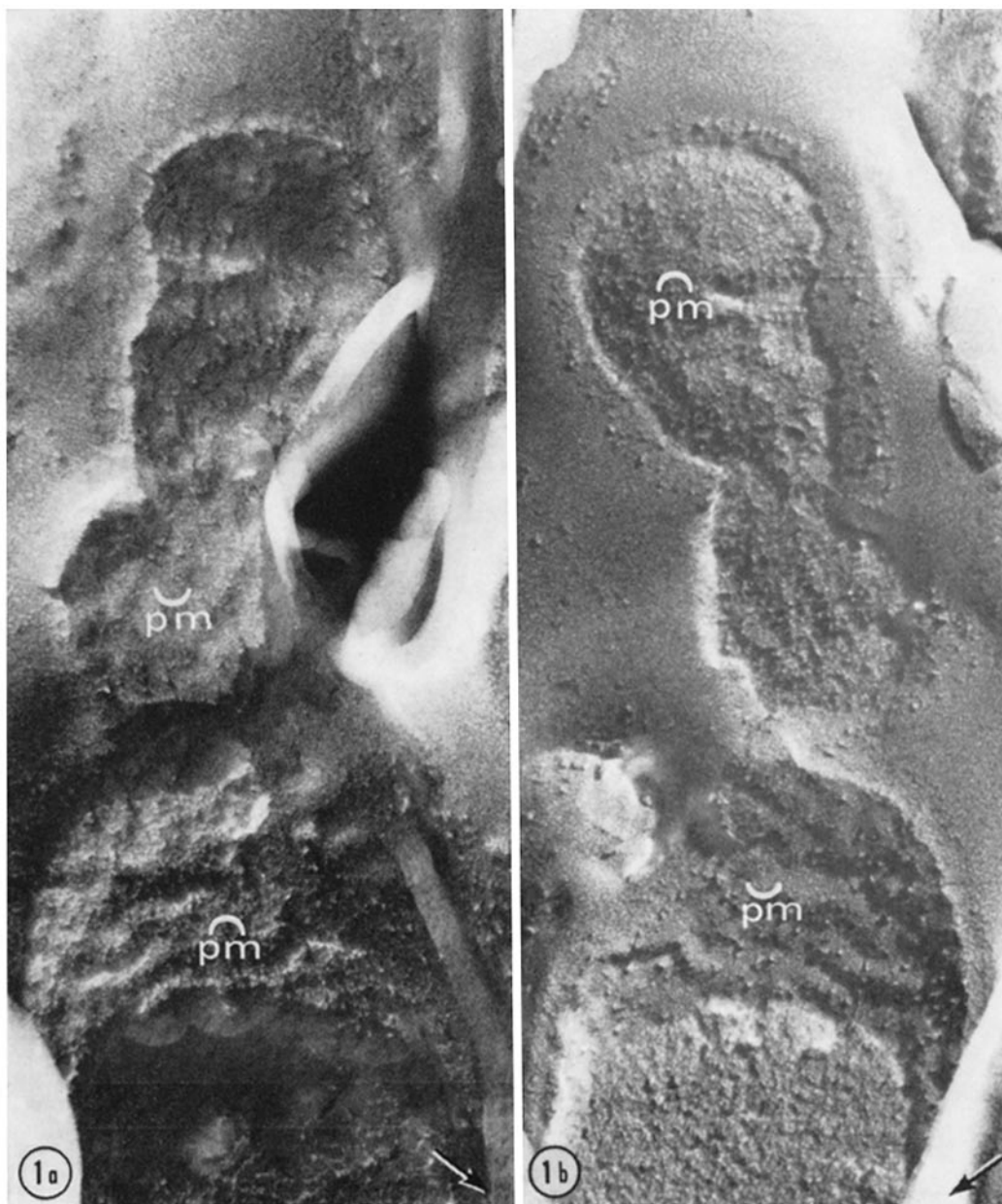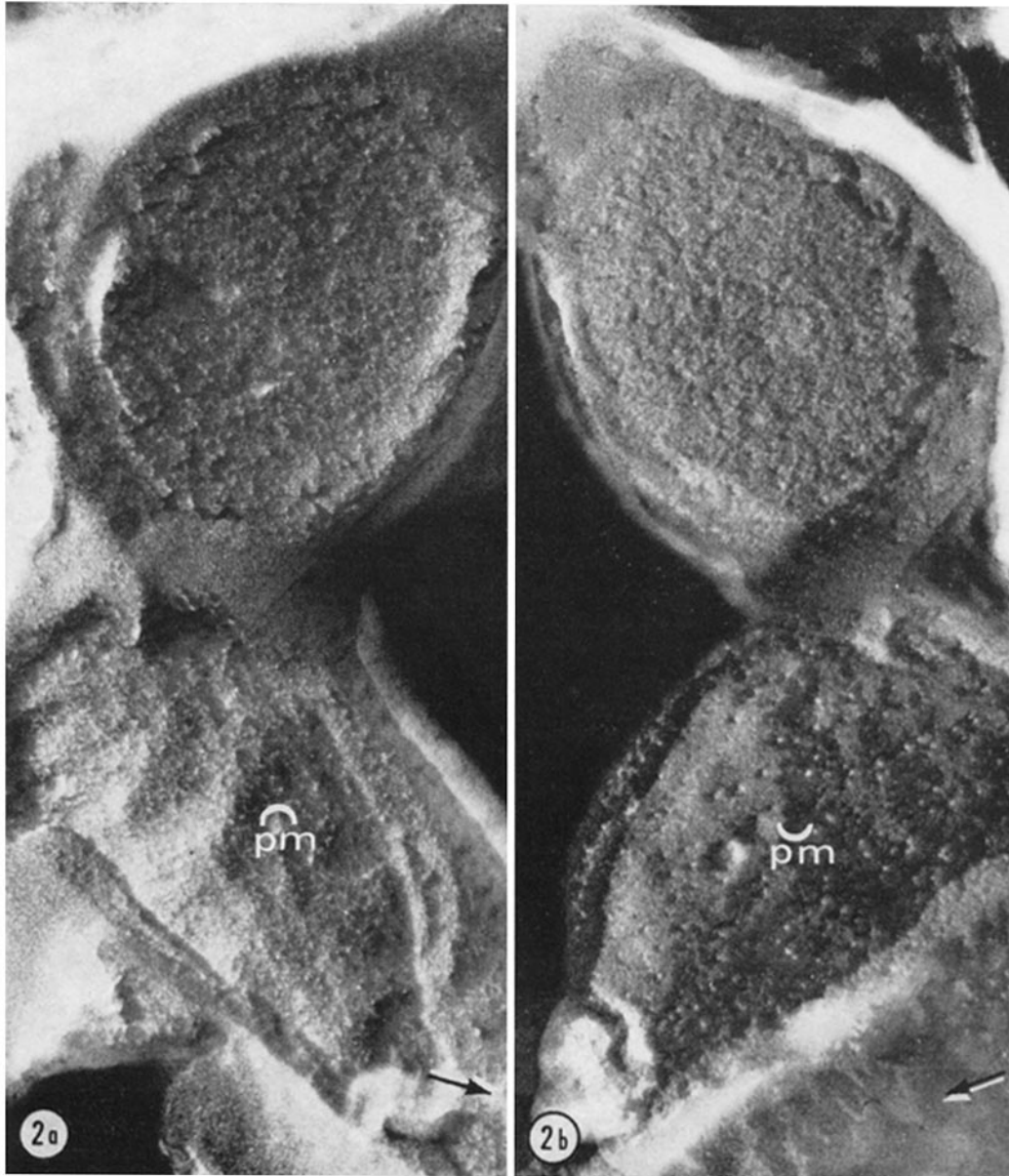

FIGURES 1 *a* and 1 *b*   Complementary freeze-fracture replicas of *B. subtilis* plasma membrane. Note that a convex face (⁀pm) with numerous particles and a concave face (⌣pm) with far fewer particles are apposed to each other. × 75,000.

apposed faces match each other closely, but the numerous particles on the convex rough face do not seem to fit into complementary holes in the apposing smooth one. This observation will be further considered in the Discussion.

Thin Sectioning of Freeze-Fractured Cells

A first concern has been to preserve the fractured cells against possible deteriorating effects of thawing. For this purpose the cells were embedded in agar, fixed in osmium tetroxide, and post-

FIGURES 2 *a* and 2 *b*   Complementary freeze-fracture replicas of *B. subtilis*. The two surfaces of a cross-fractured cell are shown (upper parts of figures); a convex rough face (p̂m) and its concave smooth counterpart (p̃m) are visible (lower parts of figures). × 75,000.

treated with uranyl acetate (12, 16). We have shown before that the fracturing properties of the plasma membrane do not alter after this particular chemical fixation (12). After thawing, the fractured agar blocks were dehydrated in a graded series of acetone and embedded in Vestopal W (16). Sections were cut normal to the original fracture plane. Fig. 3 shows the edge of a fracture plane (fs) together with the edge of the section (es). In the upper left of Fig. 3 (arrows) some freeze-fractured bacteria are visible. For the analysis of the course of the fracture plane, it is required that the section pass normal to the cell envelope in order to reveal clear-cut, electron-opaque profiles. Such an example is shown in Fig. 4 *a*, whereas more detail can be seen in Fig. 4 *b*. It appears that upon removal of part of the cell content (fracturing along a concave face) the outer profile of the plasma membrane (opm) remains visible. This suggests that the fracture plane does not run between cell wall and plasma membrane but somewhere between the outer leaflet of the plasma membrane and the cyto-plasm (see Fig. 6). The reader should notice that the bacterial fragments are well preserved and that the cleanness of the fractured surface is especially striking.

In order to study further the course of the fracture plane, we had to examine cells in which part of the cell envelope was broken away (fracturing along a convex face). In contrast to the first case (concave fracturing, Figs. 4 *a* and 4 *b*), this proved to be more difficult. As is well known from *B. subtilis* (Fig. 1 in reference 20), the inner profile of the plasma membrane barely shows up against the bordering cytoplasm. An example of a favourably convex fractured cell is shown in Fig. 5 *a*. Here the profile of the cell wall and the outer profile of the plasma membrane stop at the site where the fracture enters the cell envelope. A faint trace of the inner profile may be discerned (arrows, Fig. 5 *b*). It can also be seen here that, upon fracturing, the outer profile of the plasma membrane disappears together with the cell wall, indicating again that there is no fracture plane between wall and membrane.

DISCUSSION

In the present study we have demonstrated the existence of *one* fracture plane belonging to the plasma membrane of *B. subtilis* by means of complementary replicas. The course of the fracture plane has been traced in thin sections of freeze-fractured bacteria.
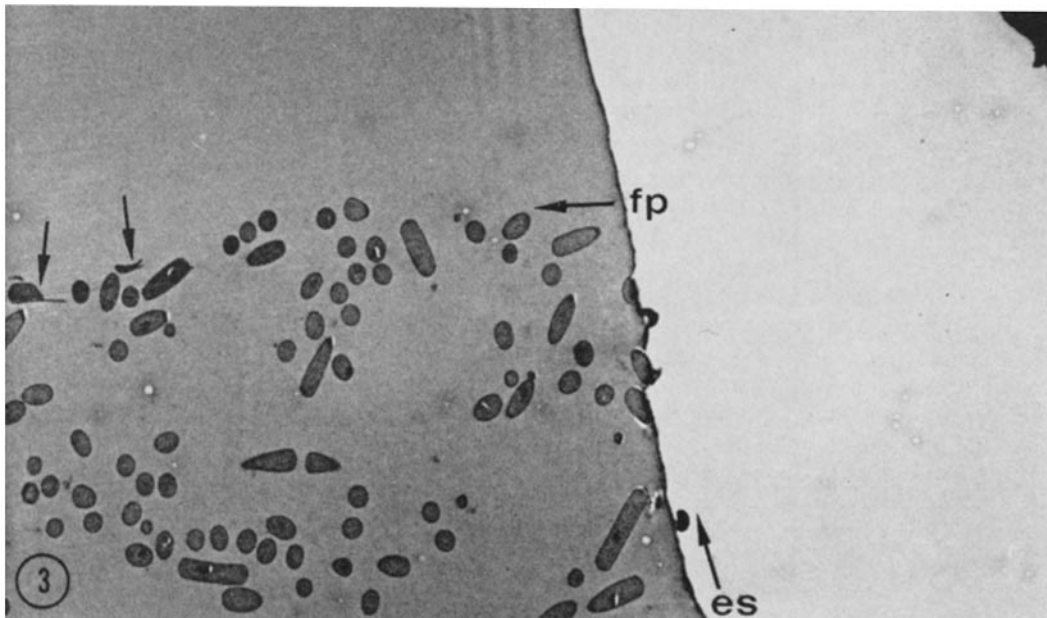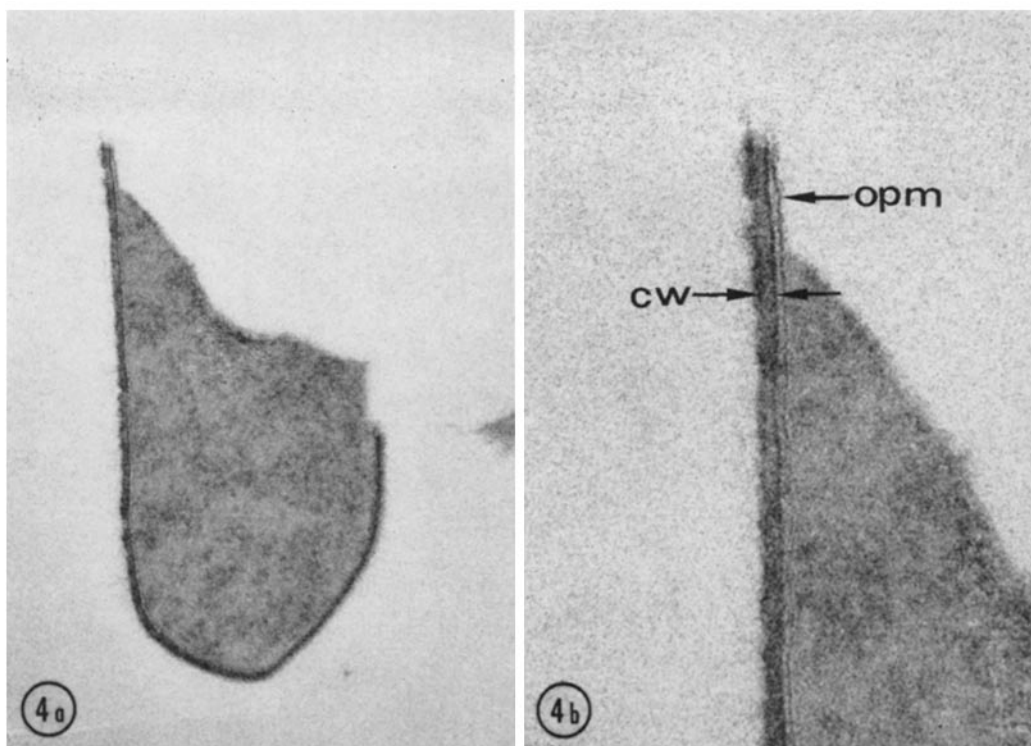

FIGURE 3   Freeze-fractured and subsequently embedded and sectioned bacteria. Note edge of thin section (*es*), fracture plane (*fp*), and freeze-fractured bacteria (arrows). × 45,000.

Figures 4 a and 4 b   A concave freeze-fractured cell in which part of the cell content has been removed (Fig. 4 a). More detail is visible in Fig. 4 b. The cell wall (cw) and the outer profile of the plasma membrane (opm) did not separate upon fracturing. X 75,000 and 200,000, respectively.

Uniqueness of the Fracture Plane

The previous observations of only two faces belonging to the plasma membrane of B. subtilis (11, 15) suggest the presence of one fracture plane (6). A direct proof for this in B. subtilis has now been given by means of complementary replicas, which show that the convex rough face and the concave smooth face are apposed to each other. However, the numerous particles on the convex face do not seem to fit into corresponding holes in the concave face. This appears to be a general phenomenon and several explanations are possible; for instance: (a) fracturing results in plastic deformation of the exposed structures (6); (b) some material disappears upon fracturing (14); (c) the structures are modified by contamination (7); and (d) shadow-casting obscures the exposed structures: i.e., the particles become larger and the holes become smaller and thus do not seem to fit. This latter possibility is not unlikely since high resolution shadow-casting of the erythrocyte

membrane revealed numerous pits in the smooth face which were presumed to match the particles on the complementary rough face (R. L. Steere, personal communication). However, too little is known about the physical aspects of freeze-fracturing and the chemical nature of the observed structures to permit a definite conclusion regarding this matter.

Location of the Fracture Plane

The presence of one fracture plane does not necessarily imply that the fracture plane runs inside the membrane. Three possibilities for the location of the fracture plane are shown in Fig. 6: (a) between cell wall and plasma membrane; (b) inside the membrane; and (c) between membrane and cytoplasm. A combination of these possibilities does not seem likely with respect to the plasma membrane of B. subtilis. Possibility (a) can be excluded because the cell wall and the outer profile of the plasma membrane are re-
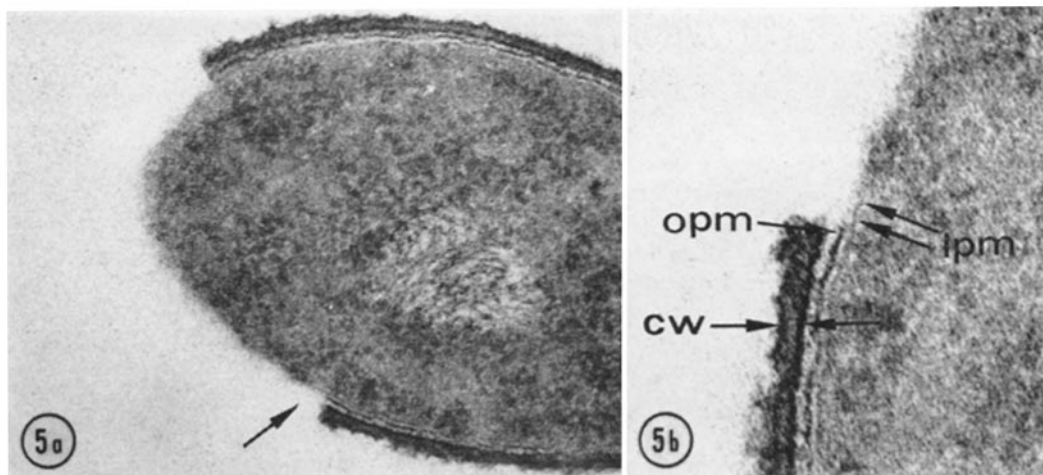
FIGURES 5 a and 5 b   A convex freeze-fractured cell. The part indicated by an arrow in Fig. 5 a is shown in more detail in Fig. 5 b. The cell wall (cw) and the outer profile of the plasma membrane (opm) have been removed together. A faint trace of the inner profile of the plasma membrane (ipm) may still be discerned. × 110,000 and 200,000, respectively.
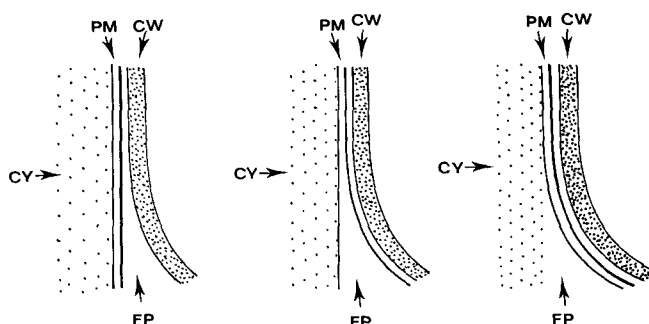

FIGURE 6   Schematic representation of fracture possibilities with respect to the plasma membrane of *B. subtilis. Left*, the fracture plane runs between cell wall and plasma membrane; *center*, the fracture plane splits the plasma membrane; and *right*, the fracture plane runs between plasma membrane and cytoplasm. *FP*, fracture plane; *CW*, cell wall; *PM*, plasma membrane; *CY*, cytoplasm.

moved simultaneously upon fracturing (Figs. 4 and 5). In this case the stability of the presumed monolayer (opm, in Fig. 4 b) is remarkable. It has probably been anchored firmly to the cell wall by chemical fixation. A conclusion with respect to alternatives (b) and (c) can be made less firmly on the basis of fractured and subsequently sectioned profiles alone. Two explanations are possible: (1) the general poor visibility of the inner line of the strongly asymmetric profile of the plasma membrane, and/or (2) its instability after thawing. We feel, however, that possibility (c) is less likely, first, because a trace of the inner profile of the plasma membrane can still be dis-

cerned after convex fracturing in favourable sections (Fig. 5); and second, because fracturing along the cytoplasmic side may result in an etchable face which so far has not been observed in our specimens. Alternative (b) thus seems most plausible and it gives further support to the concept of membrane splitting (1, 2).

It should be noted that our results with fractured and subsequently sectioned membranes are quite different from those of Leak (8) and Bullivant and Weinstein (4). These authors interpreted their observations as supporting the idea that membranes expose their outer surfaces upon fracturing. The difference in results is probably due

to the fact that we employed osmium tetroxide fixation *before* fracturing, which might have better preserved the fractured membrane parts (presumably monolayers) after thawing. Material might have been leaking out of their preparations (4, 8), creating the possibility of exposing non-fractured membranes near the original fracture plane in thin sections. In a later study Bullivant (3) observed occasionally "half a unit membrane" in sections if the original fracture face were protected with a layer of evaporated carbon.

In an earlier publication we showed that what was then interpreted as outer surface, but which is shown in these experiments to, in fact, be inner surface, is well preserved after osmium tetroxide fixation even if followed by extensive dehydration in acetone (12). As a contrasting agent, osmium tetroxide appears to be most abundantly present in the outer membrane region, notably at the cell wall side. In this connection it is important to note, as stressed by Stoeckenius and Engelman (18), that fixation and staining are not identical processes.

It further appears that an advantage of fracture-plane–sectioning, described above, as compared with deep-etching (14, 19), lies in the possibility of relating the fracture plane directly to the triple-layered image of the membrane in thin sections. Particles visible *on* membranes after negative staining, on the other hand, cannot be directly related to the particles on freeze-fractured membrane faces, since the latter are in the membrane interior (see our Discussion in reference 11).

In conclusion, it can be said that the present results permit a better interpretation of the structural features of the freeze-fractured plasma membrane of *B. subtilis*, i.e., there is a unique fracture plane which most likely runs inside the membrane.

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