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Specific Location of Penicillin-Binding Proteins within the Cell Envelope of *Escherichia coli*

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This communication deals with the location of penicillin-binding proteins in the cell envelope of *Escherichia coli*. For this purpose, bacterial cells have been broken by various procedures and their envelopes have been fractioned. To do so, inner (cytoplasmic) and outer membranes were separated by isopycnic centrifugation in sucrose gradients. Some separation methods (Osborn et al., J. Biol. Chem. 247:3962–3972, 1972; J. Smit, Y. Kamio, and H. Nikaido, J. Bacteriol. 124:942–958, 1975) revealed that penicillin-binding proteins are not exclusively located in the inner membrane. They are also found in the outer membrane (A. Rodríguez-Tébar, J. A. Barbas, and D. Vásquez, J. Bacteriol. 161:243–248, 1985). Under the milder conditions for cell rupture used in this work, an intermembrane fraction, sedimenting between the inner and outer membrane, can be recovered from the gradients. This fraction has a high content of both penicillin-binding proteins and phospholipase B activity and may correspond to the intermembrane adhesion sites (M. H. Bayer, G. P. Costello, and M. E. Bayer, J. Bacteriol. 149:758–769, 1982). We postulate that this intermembrane fraction is a labile structure that contains a high amount of all penicillin-binding proteins which are usually found in both the inner and outer membranes when the adhesion sites are destroyed by the cell breakage and fractionation procedures.

Penicillin-binding proteins (PBPs) are minor proteins located in the cell envelope of bacteria. These proteins are actually enzymes which catalyze the last stages of peptidoglycan biosynthesis (see reference 34 for a review). Over the last 9 years, considerable efforts have been made by a number of laboratories to elucidate the enzymic properties of the PBPs. However, not much attention has been paid to the location of these proteins within the cell envelope, despite its relevance in the cell cycle processes. Indeed, two main processes of the bacterial life cycle, namely, elongation and cell division, are accompanied by and, to a great extent, facilitated by drastic changes in peptidoglycan structure. PBPs are greatly responsible for such changes. Obviously, cell division occurs, under normal circumstances, at a specific place on the cell envelope. Available data also indicate that elongation of the peptidoglycan sacculus also takes place at discrete points (25, 27). These data heighten the importance of the research into PBP localization. PBPs from *Escherichia coli* have been considered for a number of years as cytoplasmic membrane proteins (33). This statement was made on the basis of the solubility of PBPs in sodium lauryl sarcosinate (Sarkosyl), because it was thought at one time that proteins from the outer membrane (OM) were all Sarkosyl insoluble, whereas proteins in the inner membrane (IM) could be solubilized by 1% (wt/vol) Sarkosyl (11). However, late work showed that some minor proteins from the OM could be also solubilized by Sarkosyl (9). One of the reasons why major OM proteins are not solubilized by Sarkosyl is the tight attachment of those structures to peptidoglycan (2). Both OmpA and OmpF can be solubilized by 1% (wt/vol) Sarkosyl after digestion of peptidoglycan by lysozyme (Barbas et al., submitted for publication).

Recent work from our laboratory (23, 24) has demonstrated that with standardized methods for OM and IM separation (20, 21) a significant amount of PBP material was found associated with the OM. Methods for separation of IM and OM by isopycnic centrifugation in sucrose gradients (13, 19–21, 26, 31) have been extensively used for location of membrane proteins (see reference 30 for a review). In our previous work (23, 24) we applied only one method for membrane fractionation for the study of PBP location. In the present article we have extended our previous studies by using other methods for membrane separation in sucrose gradients. Our data lead us to believe that PBP material resides in discrete membranaceous structures that are easily destroyed during the separation procedure.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* PAT84 (K-12, F⁻ *thr-1 leu-6 thi-1 argH1 thy his-1 trp-1 str-9 lacY malA1 xil-7 mtl-2 tonA2 supE44 dapA lysA ftsZ*) (22) was used throughout this work. Cells were grown at 30°C in L medium (16) supplemented with 10 μg of 2,6-*meso*-diaminopimelic acid per ml and 4 mg of D-glucose per ml. Cells were grown under strong aeration. They were collected in the mid-log exponential phase of growth (absorbance at 550 nm, 1.0) by rapid cooling and centrifugation at low speed.

Radioactive labeling of cell peptidoglycan. Cells were grown in glucose-L medium supplemented with only 5 μg of 2,6-*meso*-diaminopimelic acid per ml. At the beginning of the exponential phase (absorbance at 550 nm, 0.1), 2,6-[^{3}H]diaminopimelic acid was added at a final concentration of 5 μCi/ml. Cells were allowed to grow and were harvested as described below.

Isolation of cell envelopes and separation of IM and OM. The following procedures were used.

(i) Method A. The method of Osborn et al. (20) was first developed for fractionation of *Salmonella typhimurium* envelopes. After EDTA-lysozyme treatment, spheroplasts were broken by osmotic shock or sonication. For osmotic shock, spheroplasts suspended in 10 mM Tris hydrochloride (pH 7.8)–0.25 M sucrose–1 mM EDTA–33 μg of egg white

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lysozyme per ml were diluted 10 times with cold 10 mM Tris hydrochloride buffer (pH 7.8) with gentle stirring. Unbroken cells were removed by centrifugation at 5,000 × *g* for 10 min at 4°C. Further steps were performed as described previously (24). Full details for sonication are given below.

(ii) Method B. The method of Bayer et al. (8) for the fractionation of *Salmonella annatum* envelopes was in its turn derived from that of Smit et al. (31). Briefly, cells from a 120-ml culture at an absorbance at 550 nm of 1.0 were collected by a short centrifugation at 4°C and then suspended in 100 ml of cold M9 medium (18) supplemented with 1 mM $CaCl_2$ and 1 mM $MgSO_4$. Cells were centrifuged as described above and suspended in the salt-supplemented M9 medium at a cell concentration giving an absorbance at 550 nm of 12. Pancreatic DNase and RNase were added to a final concentration of 40 μg of each enzyme per ml. After a short incubation at low temperature, cells were broken by one passage through a French press. After breakage of the cells the next steps for envelope isolation were carried out as described previously (8).

Procedures used for cell rupture. (i) French press. An Aminco French press was used (American Instrument Co., Silver Spring, Md.). Bacteria were pressed into a 3/8-in. (ca. 0.94-cm)-diameter cell (model 4-3399). Two different relief pressure valves were used. The valve model A72-51069 is a soft-seal valve stem with a nylon ball. The valve model A37-51069 is an all-steel plug. Unless otherwise stated, all operations were carried out at 20,000 lb/in^2. The volume of the sample subjected to pressure treatment was 4 ml. The relief pressure valve was operated by hand. Special care was taken to catch individual drops from the sample output tube. The whole operation usually took 5 to 10 min.

(ii) Ultrasonic treatment. An MSE 150-W sonicator (MSE Scientific Instruments, Crawley, United Kingdom) was used, provided with either a 1/8-in. (ca. 0.31-cm) or a 3/8-in. (ca. 0.94-cm) probe. The tip was inserted 1 cm into the sample. Samples (8 ml) were contained in a shortened 30-ml round-bottom Corex tube which was then inserted into an ice bath. Times of sonication varied and are indicated in Results and in the figure legends for particular experiments. The ultrasonic source was always operated at 1.8 μm amplitude (peak to peak), taking place at the transducer-probe interface. The actual movement of the tip of the probe was 9.9 μm for the 3/8-in. probe and 12.6 μm for the 1/8-in. probe.

Isopycnic centrifugation in sucrose gradients. For isolation of cell envelopes by method A (see above), the sucrose solutions of the gradients were prepared in 5.0 mM sodium EDTA, pH 7.4. For method B, sucrose solutions were made in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.4). The discontinuous gradients consisted of the following layers from bottom to top of the centrifuge tube: 1.0 ml of 55%, 2.2 ml of 50%, 2.8 ml of 45%, 2.5 ml of 40%, 1.7 ml of 35%, and 1.7 ml of 30% (wt/wt) sucrose. Gradients were centrifuged at 30,000 rpm in a Beckman SW40 rotor for 20 h at 2°C. After the run, gradients were dissected into 20 to 30 fractions by pumping a denser sucrose solution into the bottom of the gradient tube.

Study of membrane markers. The purity and degree of cross-contamination were assessed as follows: (i) measurement of β-NADH oxidase as a marker for the IM (in all the fractions obtained from the gradients) by published procedures (10, 21), (ii) measurement of 2-keto-3-deoxyoctanoic acid content in all gradient fractions (21), and (iii) recognition of the major OM proteins in the gradient fractions by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis.

In addition, labeled peptidoglycan (if present) was detected in the gradient fractions by precipitating samples with trichloroacetic acid, filtering through Whatman GF/C glass paper, and counting the radioactivity retained in the filter as previously described (24).

The amount of protein present in the gradient fractions was estimated either by the Folin phenol reagent method or by its absorbance at 280 nm.

Detection of PBPs. PBPs from all fractions of the sucrose gradient were detected as described previously (24). The radioiodinated Bolton and Hunter derivative of ampicillin was used as a label (24, 28). Labeled membranes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as reported earlier (14). Dried slab gels were autoradiographed as described previously (15).

Phospholipase assay. The substrate for phospholipases A_1 and A_2 was prepared by incorporating both [^{14}C]palmitic and [^{14}C]oleic acids into the phospholipids of *E. coli* B. Autoclaved cells were used as a source of substrate for phospholipase. We strictly followed the procedure used by Bayer et al. (8), which is a modification of the method of Scott et al. (29). After the reaction was quenched, both hydrolyzed [^{14}C]oleic and [^{14}C]palmitic acids were separated from phospholipids by thin-layer chromatography in silica gel plates (8). After drying, the plates were exposed to iodine vapors to develop the spots corresponding to both oleic and palmitic acids (Sigma Chemical Co., St. Louis, Mo.) that had been cochromatographed as external standards. Later, the plates were briefly immersed in 25% (wt/wt) diphenyloxazole in dimethylsulfoxide, air dried, and then exposed on X-ray films. The radioactive spots, those which remained in their original location and those corresponding to the labeled fatty acids (hardly separated in this system), were cut out, and the silica gel was scraped off. Both phospholipids and fatty acids were eluted with ether, and their radioactivity was measured in a toluene-based scintillation liquid.

Radioactive chemicals. ^{125}I-labeled Bolton and Hunter reagent (2,000 Ci/mmol) and [^{14}C]oleic and [^{14}C]palmitic acids were purchased from Amersham International, Amersham, United Kingdom. 2,6-*meso*-[^{3}H]diaminopimelic acid came from the Commissariat à l'Energie Atomique, Gif-sur-Yvette, France.

RESULTS

Separation of IM and OM after rupture of the cells with a French press. For the first set of experiments, the nylon-balled relief valve was used. The results of separation of IM from OM are shown in Fig. 1. Not only most PBP material but also most protein components appeared in the gradient in a position corresponding to IM. The amount of PBP material related to the amount of total protein was very similar in both fractions (data not shown). Each PBP was evenly distributed in both IM and OM, and no selectivity in the distribution of PBP 3 was found (see below). Parallel experiments were also done after breaking the cells at either 12,000, 9,000, 6,000, or 3,500 lb/in^2. Results were identical to those displayed in Fig. 1 and are therefore not shown.

We believe that the passage of the cell suspension through the valve orifice produced a severe extrusion of most proteins from the OM structures, which are partly formed by peptidoglycan and lipopolysaccharide, and are precisely those which confer a higher density on the OM. The amount of protein collected in association with the OM represented about 10% of the total envelope protein. Obviously these data are not consistent with most reported studies dealing with separation of IM and OM (5).

When the whole steel plug valve (model A37-51069) was used for cell breakage, very different patterns of membrane separation were obtained. We carried out a series of nine distinct experiments. In all the experiments we obtained an adequate separation of IM from OM. The distribution of membrane markers revealed that cross-contamination was never higher than 5%. However, the distribution of the PBPs in the envelope fractions varied strikingly as described in detail below.

In four experiments the separation of the two membranes and the distribution pattern of PBPs resembled that reported in our previous study with a different method of envelope fractionation (24) (Fig. 2). The main difference found was that PBP 3 appeared evenly distributed in both IM and OM. PBP 7b was only found in the IM, whereas PBP 7a appeared in both membranes. PBP 7b is derived from PBP 7a by proteolysis of PBP 7a (Lacat et al., submitted for publication).

In three experiments of this series, the separation of both IM and OM was also clear (Fig. 3). A very small intermediate band (intM, obtained at the 40%–45% sucrose interface) was also obtained. This band contained less than 5% of the total membrane protein and only around 4% of the β-NADH oxidase activity found in the total cell envelope. Nevertheless, more than 15% of the total PBP material was found associated with the intM. When related to the amount of protein present, the quantity of PBP molecules in the intM was considerably higher than in both the IM and the OM.

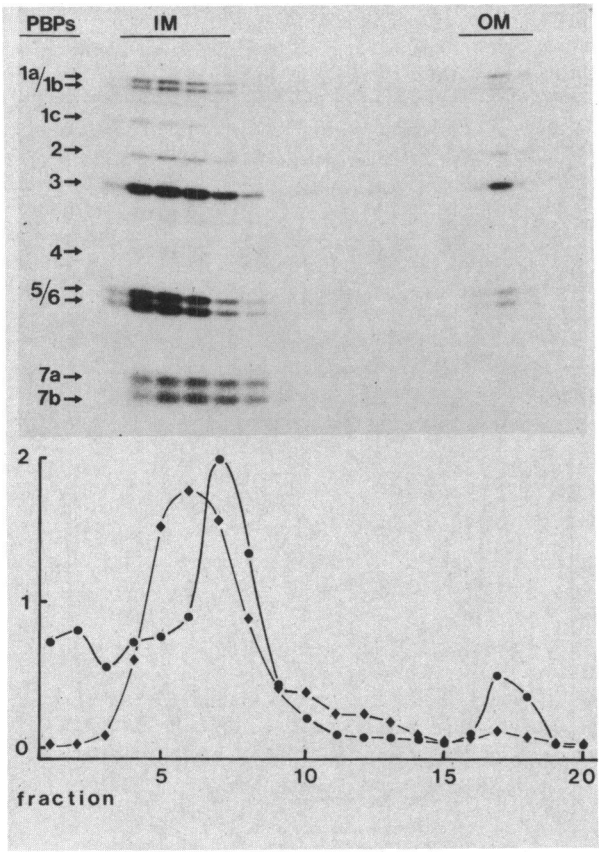

FIG. 1. Distribution of PBPs in envelope fractions. Cells were harvested and treated for envelope fractionation as described previously (8). Bacteria were broken by one passage through the French press at 20,000 lb/in^2. The nylon-balled pressure relief valve was used. Twenty-five fractions were collected from the sucrose gradient. Samples from each fraction were used for analyzing PBPs (gel autoradiography at the top of the figure). Symbols: (●) protein content determined by the Folin phenol reagent method, (◆) β-NADH oxidase activity. The ordinate is in arbitrary units. The band identified as PBP 7 in this work is now known to be the same protein as PBP 8 in our previous work (23, 24), since the possibility that some *E. coli* strains might have a PBP migrating between PBPs 6 and 8 is rather unlikely (Lacat et al., submitted for publication).

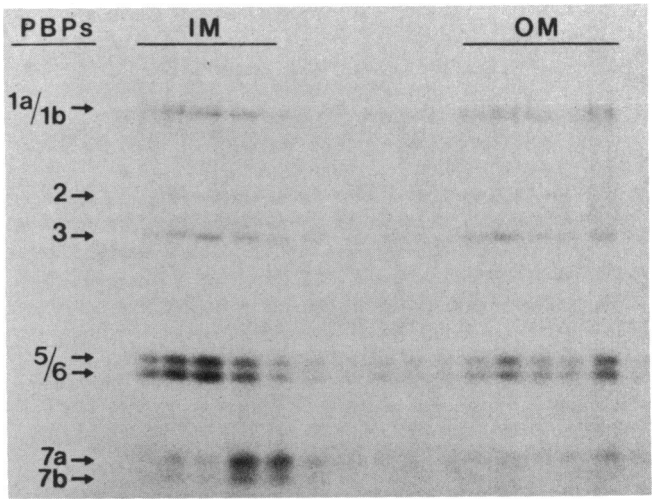

FIG. 2. Distribution of PBPs in envelope fractions. Cells were broken in a French press with a whole steel pressure relief valve. Fractions from sucrose gradient were analyzed for PBP content. See the text for additional data and also the legends of Fig. 3 and 4 for comparison.

In two of our experiments, results were even more dramatic. More than half of all PBP material was found in the intM (Fig. 4), despite the fact that the intM contained only a small amount of protein and β-NADH oxidase activity. Phospholipase activity from the envelope fractions was measured in these two experiments. For this purpose, fractions corresponding to either IM, intM, or OM were pooled, diluted in water, and centrifuged at 200,000 × *g* for 2 h at 2°C. Pelleted membranes were suspended in an equal volume of 100 mM Tris hydrochloride buffer (pH 7.5). A sample was used for the enzyme assays, which were carried out as fully described in Materials and Methods. Phospholipase activity was absent from the IM and was distributed in both the intM and the OM at a ratio of 40:60, respectively.

Separation of IM and OM from spheroplasts. The method of Osborn et al. (20) was followed with some variations concerning the length of the time of sonication. We followed the standard method used previously (24) and confirmed our previous data for the experiment shown in Fig. 5. After forming spheroplasts, the cells were subjected to a 30-s pulse of sonication with the 3/8-in. probe. There was a very clear resolution of both IM and OM. The intM was nearly absent, with no significant amount of PBP material. All PBPs except PBPs 3 and 7 were found evenly distributed in both IM and OM. PBPs 3 and 7b were mostly found in the IM. For the experiment shown in Fig. 6, the time of sonication was 10 s, but the 1/8-in. probe was used. Again the separation of IM and OM was clear, but this time an intM fraction was obtained. The enrichment of PBPs in the membrane fractions related to the amount of protein measured in all of the fractions from the gradients can be seen. It is clear that this intM fraction is again particularly enriched in PBP material. In addition, PBP distribution in both IM and OM was similar to that observed by the standard method.

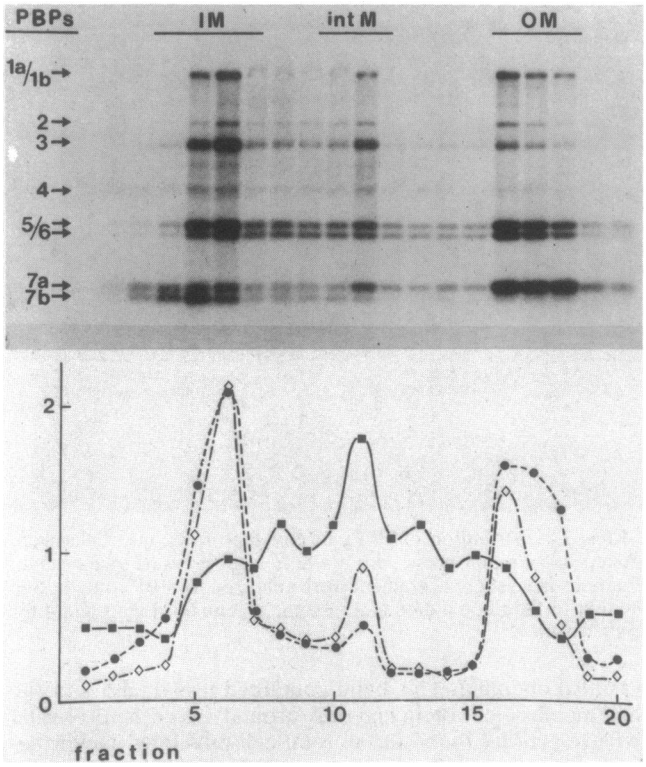

FIG. 3. Distribution of PBPs in envelope fractions. Cells were broken by pressure treatment in a French press with a whole steel pressure relief valve. Samples from each of the 25 fractions collected from the sucrose gradient were used for detection of PBPs and for measuring protein content by the Folin phenol reagent method (●). To quantify PBPs, each lane from the dried polyacrylamide slab gel was cut out after exposure, and the radioactivity corresponding to all PBPs (except PBPs 7a and 7b) was measured in a gamma counter (◇). The square symbols (■) give the radioactivity related to the protein content in each fraction. Compare with Fig. 2 and 4.

Figure 7 shows a membrane separation pattern after sonicating the spheroplasts for 5 s using the 1/8-in. probe. The separation pattern was deficient, because sonication did not separate IM from OM. The OM was heavily contaminated by β-NADH activity. The failure of the separation appears to result from the lack of a proper detachment of most IM from the OM structures, since both fractions migrated toward the denser layers in the sucrose gradients.

From the results presented above, one can conclude that the method used for cell breakage may not be too critical for the discrimination of most proteins associated with either the IM or the OM, but it is crucial to the study of the location of PBPs. We carried out a set of 15 experiments in which spheroplasts were sonicated for 8 s with the 1/8-in. probe. In most experiments, results were similar to those shown in Fig. 6. In three experiments, like the one shown in Fig. 8, at least 50% of all PBP material was found associated with the intM. The lack of reproducibility suggests that it is really very difficult to preserve some envelope structures with a high content in PBP material.

We have also studied the distribution of PBPs by using a less severe method for cell rupture. Spheroplasts were prepared and lysed by osmotic shock (Fig. 9). PBPs were also present in gradient fractions of intermediate density in significant amounts.

Actually, the intM was greatly enriched in PBPs, because it contained a small amount of proteins. In our experiments, rupture of spheroplasts by osmotic shock appeared to be a less severe method for preserving the intM fraction. However, cross-contamination of β-NADH oxidase was higher (Fig. 9).

DISCUSSION

PBPs possess some special characteristics different from those of most membrane proteins from *E. coli* envelopes. They are actually membrane proteins that act as enzymes on a relatively rigid and large substrate with a rather restricted mobility. As far as it is known, peptidoglycan is closely associated with the OM, not only through covalent bonds provided by the lipoprotein, but also by tight connection, probably of an electrostatic nature, with some major OM proteins (2). In addition, residual peptidoglycan is found associated only with the OM when the envelope is fractioned (20, 21, 24) (Fig. 4). Changes in the structure of OM-linked peptidoglycan could be due to PBPs located in the neighborhood of the OM. The PBPs could be collected in association

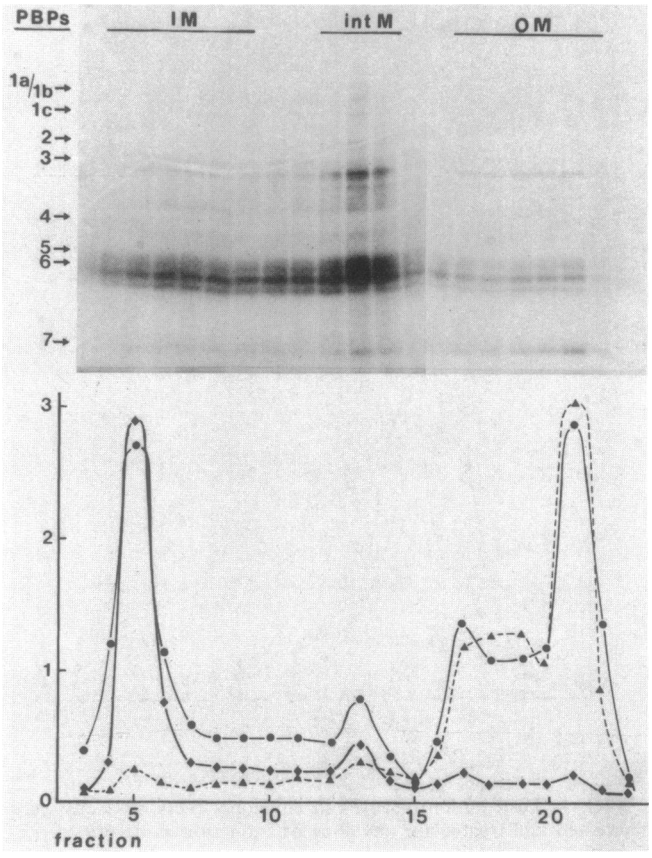

FIG. 4. Distribution of PBPs in envelope fractions. Cells were first labeled with [^{3}H]diaminopimelic acid (5 μCi/ml) at the beginning of the exponential phase. Bacteria were then broken by treatment in a French press with a whole steel pressure relief valve. Samples from each fraction of the sucrose gradient were analyzed for their PBP content, β-NADH oxidase activity (◆), protein concentration determined by the Folin phenol reagent method (●), and trichloroacetic acid-precipitable peptidoglycan as measured by its labeled diaminopimelic acid residue (▲). See the text for more details and also Fig. 2 and 3 for reference.

with the OM during the procedure for envelope fractionation.

Data presented here suggest that all PBPs may be located in special structures different from either the IM or the OM but sharing some of their characteristics. Our proposal that PBPs are preferentially located in these structures is supported by the finding that they are often found associated with the IntM fraction of the sucrose gradients. In this communication, we have chosen to present an ample survey of the results obtained by using distinct modalities of membrane separation by isopycnic centrifugation in sucrose gradients. When cells were broken by the French press with the nylon-balled valve, most components were extruded from the OM, and most protein material and PBPs were found at a gradient position corresponding to the IM. On the contrary, when cell spheroplasts were broken by a sonication pulse that was too weak, complete detachment of IM from OM was not brought about, and most protein material and most PBPs were found in the gradients at a position corresponding to the OM (Fig. 7). However, a much stronger pulse of sonication produced a definite separation of both IM and OM (Fig. 5), and PBPs appeared in both IM and OM and were absent from the intM (24). In the three cases described above, it appeared that intM structures were not preserved. However, in the rest of the experiments presented in this communication, we have actually found a clear enrichment of PBP material in the intM relative to the amount of protein found in this fraction. This enrichment was reproducible, but the absolute amounts of PBPs found in the fractions were not. The lack of reproducibility could be due to reasons such as the difficulty in controlling the sonication burst or the existence of phospholipase contaminating our commercial RNase (6) which might damage the intM. But the lack of reproducibility was most likely due to the extreme lability of the intM structures, possibly possessing a metastable nature that may be correlated with Bayer junctions.

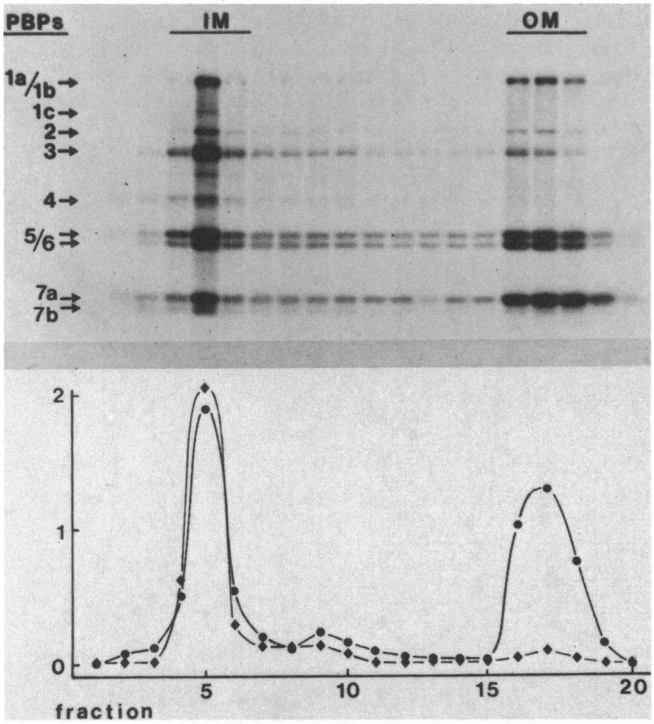

FIG. 5. Distribution of PBPs in envelope fractions. Cells were broken and envelopes were fractioned by the method of Osborn et al. (20). Spheroplasts were sonicated for 30 s using a 3/8-inch probe. Envelopes were fractioned in an EDTA-containing sucrose gradient. Samples from each fraction were used for studying PBPs, protein (●) and β-NADH oxidase activity (◆).

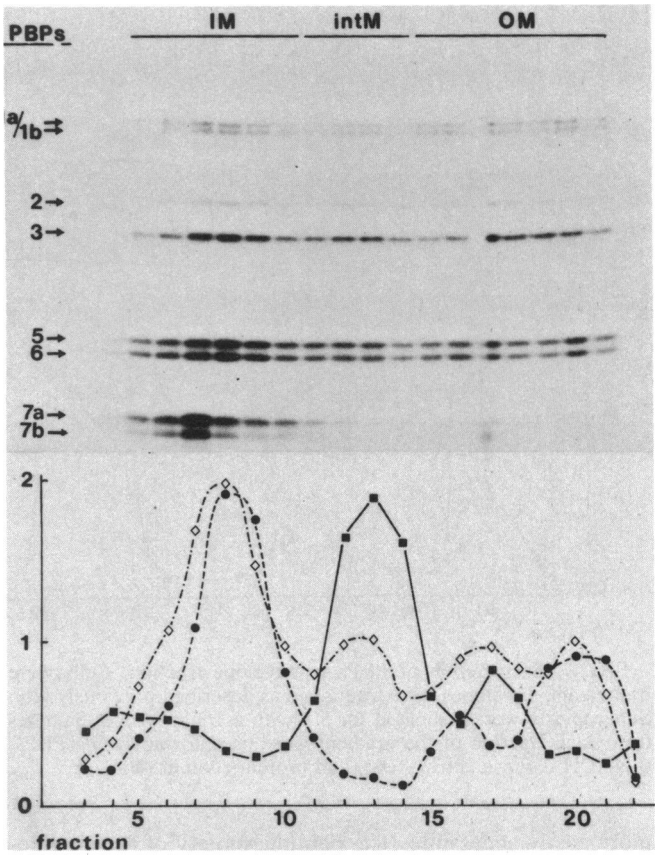

FIG. 6. Distribution of PBPs in envelope fractions. Cells were broken as described previously (20). Spheroplasts were initially formed and broken by sonication for 10 s with a 1/8-in. probe. Fractions were collected from the gradient, and samples were taken to measure PBP content and total amount of protein by the Folin phenol reagent method (●). After exposure, the dried polyacrylamide gel was cut out, and the radioactivity corresponding to all PBPs (except PBPs 7a and 7b) was measured in a gamma counter (◇). The square symbols (■) indicate the ratio of radioactivity to protein content in each fraction. The ordinate is in arbitrary units.

The existence of adhesion sites between the IM and the OM (Bayer junctions) is widely accepted (3–5, 8, 17, 32). These sites, probably of a chimeric nature, are the pathways used by the nascent lipopolysacchride and probably by some proteins travelling to the OM. The adhesion sites have been biochemically characterized to some extent by Bayer et al. in both *S. annatum* (8) and *E. coli* (7). Gradient fractions of intermediate density were found to be qualitatively different from other envelope fractions in their lipid composition and lipase activities (7). The existence of PBPs in an intermediate fraction has been also reported (23). The intM shown in Fig. 4 contained 40% of phospholipase activity. This enzyme is considered as a marker for both the OM and the IntM (7, 8).

We wish to put forward some phenomenological considerations that might suggest the physiological advantages of intM location of PBPs. If PBPs were located in an intermembrane fraction, their peptide substrates would be

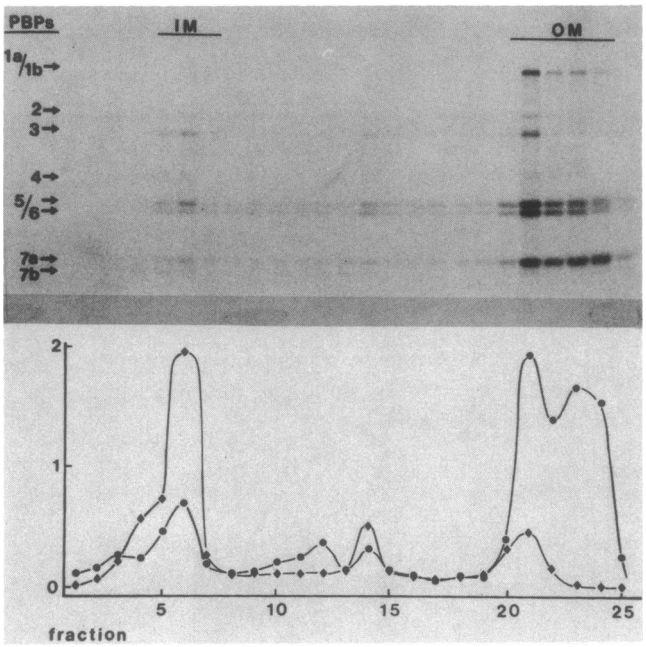

FIG. 7. Distribution of PBPs in envelope fraction. Cells were broken and envelopes were fractioned as described previously (20). Spheroplasts were sonicated for 5 s with a 1/8-in. probe. Samples from each fraction of the gradient were used for analyzing PBPs, β-NADH oxidase activity (◆), and protein content (●).

more easily accessible. The peptidic moiety of the peptidoglycan should be oriented around all of the axes of the glycan chains, which in turn must be twisted (1). Peptidoglycan is possibly a hydrated gel filling most of the periplasmic space (12). Discontinuities of the peptidoglycan surface could afford connection points between both membranes. Otherwise, it is not easy to explain how some components of a hydrophobic nature such as the lipopolysaccharide core or OM proteins can travel to the OM through a thick hydrated layer (12). Furthermore, the places where peptidoglycan is biosynthesized just where it grows could be discontinuities of the layer that allow new insertions.

Studies of the insertion of new murein revealed that this process takes place at discrete points (25, 27). When bacteria start growing, the preferential insertion of de novo peptidoglycan is just in the middle of the cell body. As long as the cell elongates, de novo peptidoglycan also appears at two additional side sites which will, in turn, be just the middle points of the future daughter. If it is assumed that PBPs appear just where macromolecular peptidoglycan emerges, it becomes obvious that at least the active PBPs are gathered together at discrete places of the cell envelope.

Our data suggest that PBPs could be gathered at special zones of the envelope where peptidoglycan metabolism is particularly active for both elongation and septation and that peptidoglycan-forming enzymes may be contained in special envelope fractions located between the two main membranes. Our results are consistent with the recent report of

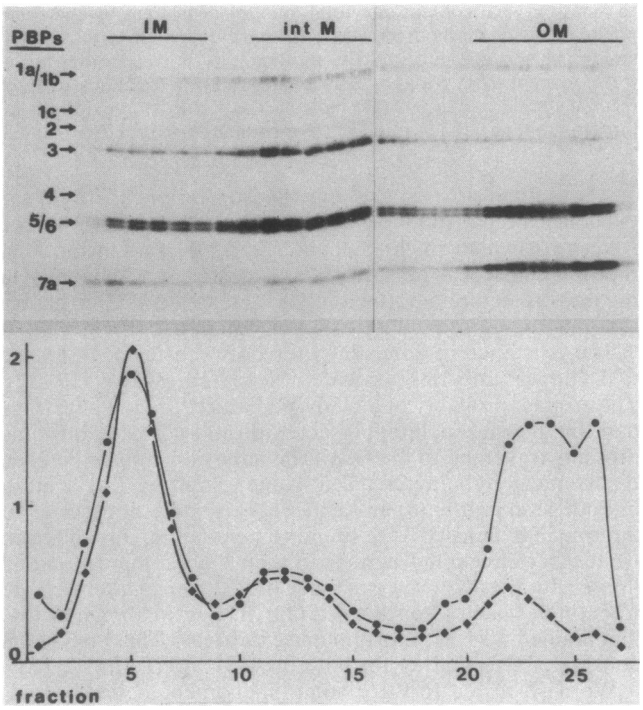

FIG. 8. Distribution of PBPs in the cell envelope. Spheroplasts were broken by an 8-s pulse of sonication with a 1/8-in. probe. The rest of the conditions are as indicated in the legend of Fig. 7.

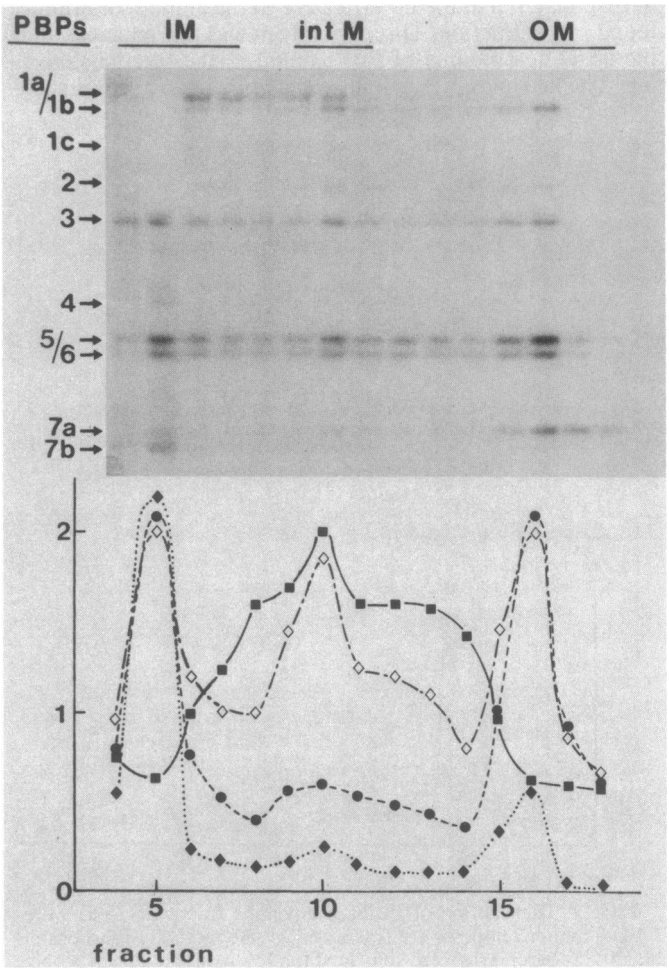

FIG. 9. Distribution of PBPs in envelope fractions. Cells were broken by osmotic shock after spheroplasting (20). The rest of the conditions and symbols are as indicated in the legend for Fig. 6.

MacAlister et al. (17), showing that in serial cuts of bacterial cells at least some intermembrane adhesion sites are not punctual zones, but real lines surrounding the cell body as a belt. These annuli are first formed at the middle of the cell body and will later act as boundaries of the septation site. Newborn periseptal annuli start forming at the two side locations where the future daughter cells will divide. Furthermore, de novo labeled peptidoglycan appears first in the intM fraction (M. E. Bayer and M. H. Bayer, 13th International Congress of Microbiology, Boston, abstr. no. P 48:6, 1982) and rapidly shifts toward the OM. Therefore, we propose that PBPs are mainly located at special zones of the cell envelope previously described as intermembrane adhesion sites (8) or periseptal annuli (17).

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