JOURNAL OF BACTERIOLOGY, Oct. 1988, p. 4775–4783
0021-9193/88/104775-09$02.00/0

Vol. 170, No. 10

Differences in Penicillin-Binding Proteins of *Streptococcus pyogenes* and Two Derived, Stabilized L Forms

OFRA LEON AND CHARLES PANOS*

Department of Microbiology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Received 11 May 1988/Accepted 8 July 1988

The penicillin-binding proteins (PBPs) of *Streptococcus pyogenes* and two of its derived, stabilized (i.e., nonreverting) L forms, an osmotically fragile L form and a physiologic isotonic L form, were compared. The numbers of PBPs in the membranes of these organisms were 6, 4, and 2 for the coccus and the osmotically fragile and physiologic isotonic L forms, respectively. Likewise, the relative amounts of total PBPs were 1.00: 1.48:0.32 for this coccus and the osmotically fragile and physiologic isotonic L forms, respectively. The two largest PBPs (PBPs 1 and 2) of the coccus were absent in both L forms, while the smallest PBPs (PBPs 5 and 6) were found in all three membranes. Deacylation (half-life) of three of the four PBPs in the osmotically fragile L form membrane required a significantly longer time than did deacylation of these presumed identical enzymes in the parental coccal membrane. Conversely, there was no such difference between the only two PBPs of the physiologic isotonic L form and the same coccal membrane proteins. Intact cells of all three organisms secreted PBPs and what appeared to be penicilloic acid and a minimal amount of free penicillin. A greater amount of these PBPs was secreted by both L forms than by the coccus. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns and ratios of secreted PBPs were identical to those from labeled membrane preparations. These differences are correlated with some of our previous findings and are discussed in terms of inhibition of cell wall synthesis and resulting membrane changes in these two derived, stabilized coccal L forms.

A considerable body of information has been gathered on the inability of stabilized L forms of *Streptococcus pyogenes* to synthesize a rigid bacterial cell wall. These studies have included morphological, biochemical, and enzymological pursuits using L forms requiring a hypertonic environment for growth as well as those able to grow on all physiologic isotonic media which support growth of the parental coccus. Some of these studies have been recently reviewed (14). An earlier study had focused on the defective synthesis of lipid intermediates for peptidoglycan in an L form of this coccal pathogen. It concluded that the inability of this L form to synthesize a cell wall in vivo was, in part, due to its failure to form significant quantities of the lipid substrates needed for peptidoglycan formation. But evidence indicated that this lesion might also be at the enzymological level (15).

Penicillin-binding proteins (PBPs) are considered penicillin-sensitive bacterial enzymes involved in terminal stages of cell wall formation (1, 5, 17, 18, 21). To our knowledge, a detailed study aimed at documenting differences in these particular enzymes in a coccal pathogen and its resulting L forms had not been documented previously. Therefore, this study compares changes in these PBPs within a nephritogenic *S. pyogenes*, type 12, and its stabilized osmotically fragile and physiologic isotonic L forms. These findings substantiate an earlier belief (15) that an enzymological lesion exists in the membrane of the L form. Also, this defect may very well be a significant contributing factor in the inability of these coccal L forms to resynthesize a rigid bacterial cell wall.

MATERIALS AND METHODS

Organisms and media. *S. pyogenes* type 12 and two of its derived, stabilized (i.e., nonreverting) L forms were used: the osmotically fragile L form, which requires a final concentration of 3.5% (wt/vol) NaCl for growth; and the physiologic isotonic L form, which grows in all media with 0.85% (wt/vol) NaCl. All cells were grown in 2.8% (wt/vol) brucella broth (Pfizer Diagnostics, Brooklyn, N.Y.) with the appropriate concentrations of NaCl for growth of the L forms and with and without 0.8% (wt/vol) bovine serum albumin fraction V (Armour Pharmaceutical Co., Chicago, Ill.). All cells were incubated at 37°C and harvested at their mid- to late logarithmic phases of growth (see below).

Growth curves. Appropriate, freshly prepared liquid media (50 ml; see above) in 125-ml Erlenmeyer flasks equipped with optically matched sidearm tubes were inoculated with an overnight culture of the coccus or derived L forms. The inoculum sizes were 5% (vol/vol) and 10% (vol/vol) for the coccus and L forms, respectively. Growth at 37°C was assessed by turbidity (optical density [OD]; Spectronic 20; Bausch & Lomb, Inc., Rochester, N.Y.) at 480 nm. Viable counts (CFUs) were performed by using the appropriate liquid media, which contained 1.5% (wt/vol) purified agar (no. 0140-01; Difco Laboratories, Detroit, Mich.). The pH of all media was 7.2. For subsequent changes in growth or survival in media without albumin, cells were obtained by centrifuging (12,000 × *g*, 10 min, 4°C) mid-logarithmically growing cultures (50 ml) of each organism and washing all cell pellets (2 × 10 ml) with their appropriate media but without albumin. Cells were then suspended in the same but prewarmed medium (50 ml), and changes in OD and CFUs were monitored with time as before.

The mid-logarithmic phase of growth and generation time for each organism were determined from growth curves of change in CFUs with time in media with albumin (see above). Under these growth conditions, the generation times for *S. pyogenes* and for both L forms were 25 and 90 min, respectively (see Fig. 3A–C).

* Corresponding author.

Membrane preparations. Late-logarithmically growing streptococcal (500 ml) and L-form (1,000 ml) cultures were harvested by centrifugation (12,000 × *g*, 10 min, 4°C) in a Sorvall centrifuge. Each cell pellet was washed twice with ice-cold 50 mM sodium phosphate buffer (pH 7.2) by centrifugation, frozen in dry ice-acetone, and stored at −80°C overnight. Cells were ruptured in an X-press (all cells from each culture suspended in 10 ml of the same buffer), with resulting >90% and 99% breakage for streptococci and L forms, respectively, as determined by light microscopy. Unbroken cells were removed by centrifugation (12,000 × *g*, 10 min, 4°C), and each supernatant fraction was subjected to high-speed centrifugation (100,000 × *g*, 30 min, 4°C) in a Spinco ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) for the collection of crude membrane preparations. Each membrane preparation was adjusted to a protein concentration of approximately 10 mg/ml in the buffer described above and stored at −80°C until needed.

Membrane penicillin-binding assay. The procedures of Dougherty et al. (3) for the detection and degree of saturation of PBPs with [^{3}H]penicillin were used with some modification. Thawed membranes (200 μg of protein) of each organism were added to different concentrations of [^{3}H]penicillin in phosphate buffer, and the final volume was adjusted to 60 μl with the same buffer. After incubation of all samples in a water bath (37°C, 10 min), the reaction was terminated by the addition of 10 μl of 20% (vol/vol) Sarkosyl with continued incubation at room temperature for 20 min. Next, 20 μl of lysis buffer (7) was added to each sample. After 3 min in a boiling water bath, all of each sample was loaded onto polyacrylamide slab gels.

SDS-PAGE. Gels were prepared and treated as described by Dougherty et al. (3) except that they were dried on a Drygel SR Slabgel dryer model SE1160 (Hoeffer Scientific Instruments, San Francisco, Calif.). Gels were stained with Coomassie brilliant blue before being dried. After drying, these PPO (2,5-diphenyloxazole)-impregnated gels were exposed to presensitized (Safelight filter, Wratten series no. 1A with 25-W white bulb at 60 cm; Eastman Kodak Co., Rochester, N.Y.; 8 s, established by trial and error) Kodak X-Omat R X-ray film (5) for from 24 to 72 h, except where noted, at −70°C. All developed films were scanned by using the Ultroscan XL laser densitometer from LKB Bromma, Sweden. Image digitization was done directly from these radioautographs of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. PBP molecular weights were determined by using SDS-PAGE molecular weight standards (catalog no. 161-0304; Bio-Rad Laboratories, Rockville Center, N.Y.) applied adjacent to the samples.

Secretion of PBP by intact coccal and L-form cells into culture medium. [^{3}H]benzylpenicillin (30 μg [0.5 μg/ml] was added to a mid-logarithmic culture (60 ml) of each organism, and incubation continued for 15 and 45 min for the coccal and L-form cultures, respectively. Cultures were then centrifuged (12,000 × *g*, 10 min, 4°C), and all cells were washed (twice) to constant specific activity with the appropriate ice-cold growth medium, without albumin, to remove residual (i.e., unbound) labeled penicillin. Cells were then suspended in the same albumin-lacking medium and returned to the incubator (37°C). Portions (10 ml) were taken immediately (zero time), and at hourly intervals, the cells were removed by centrifugation (12,000 × *g*, 10 min, 4°C) and an equal volume of cold acetone was added to the spent medium. The protein precipitate which resulted after 1 h in the cold (ice) was collected by centrifugation (12,000 × *g*, 10 min), and the pellet obtained was air dried, suspended in 50 μl of lysis buffer, and boiled for 3 min before SDS-PAGE. All fractions (i.e., cells, aqueous supernatant, acetone precipitate, and acetone-medium supernatant) from each sample were surveyed for accountability of the radioactivity initially added. Trichloroacetic acid (5% [wt/vol]) was added to all radioactive acetone-medium supernatants, and the resulting supernatant and precipitate were counted. Only the former continued to be radioactive. Also, these supernatants were reduced to near dryness by evaporation with nitrogen, and the remaining aqueous portion was removed by lyophilization. The remaining dried residue was suspended in KPO_4 buffer (1 ml), and a 60-μl portion was taken and added to 20 μl of physiologic isotonic L-form membrane preparation (prepared as described above; protein concentration, 10 mg/ml). After incubation at 37°C for 15 min, the reaction was stopped by Sarkosyl, lysis buffer was added, and the mixture was boiled in preparation for SDS-PAGE, as described above. In addition, another portion of acetone-medium supernatant was removed and treated in identical fashion except that L-form membranes (200 μg) together with labeled penicillin (0.5 μg) were added. All gels were dried, exposed to X-ray film, and analyzed as described above.

Deacylation of PBPs. Deacylation experiments were performed exactly as has been described (7) by using labeled and nonlabeled penicillin. Half-lives were calculated using a least-squares linear regression method, with a best-fit line beginning at time zero and (see Fig. 4B) including the acylation portion of the curve.

Assays, antibiotics, and reagents. Protein was determined by the method of Lowry et al. (9) by using bovine serum albumin as the standard. Freshly prepared [^{3}H]benzylpenicillin (ethylpiperidinium salt; 28 Ci/mmol) was a gift from A. Tomasz. Nonradioactive benzylpenicillin was obtained from GIBCO Laboratories, Grand Island, N.Y. Sarkosyl NL97 was purchased from ICN Pharmaceuticals Inc., Plainview, N.Y. All ingredients for SDS-PAGE were obtained from Bio-Rad Laboratories.

RESULTS

SDS gels and titration curves. Figure 1 illustrates the number of PBPs within coccal and L-form membranes and the extent of their separation by SDS-PAGE. As is apparent, the coccus contained six PBPs, whereas the osmotically fragile and physiologic isotonic L forms had only four and two PBPs, respectively. As a control, membranes from mid-logarithmically growing cultures of *S. pyogenes* in osmotically fragile L-form medium (i.e., with 3% NaCl [wt/vol]) gave the same membrane pattern and concentrations of PBPs (Fig. 1A and Table 1) as those of coccal membranes from cells grown in medium without this added NaCl.

A comparison of the titration curves of labeled penicillin binding by each of the PBPs within the membranes of the coccus and its two derived L forms as a function of antibiotic concentration is shown in Fig. 2A through C. Whereas all of the PBPs within the coccal (Fig. 2A) and physiologic isotonic L-form (Fig. 2C) membranes became saturated when titrated with labeled penicillin, PBP 6 in the membrane of the osmotically fragile L form (Fig. 2B) did not. The MIC of penicillin for the parental coccus is indicated (Fig. 2A). As expected, both L forms were insensitive to greater than 1,000 μg of this antibiotic per ml.

Properties of PBPs. Table 1 compares some of the properties of the PBPs detected within the membrane of *S. pyogenes* and two L forms. The PBPs of the coccal membrane

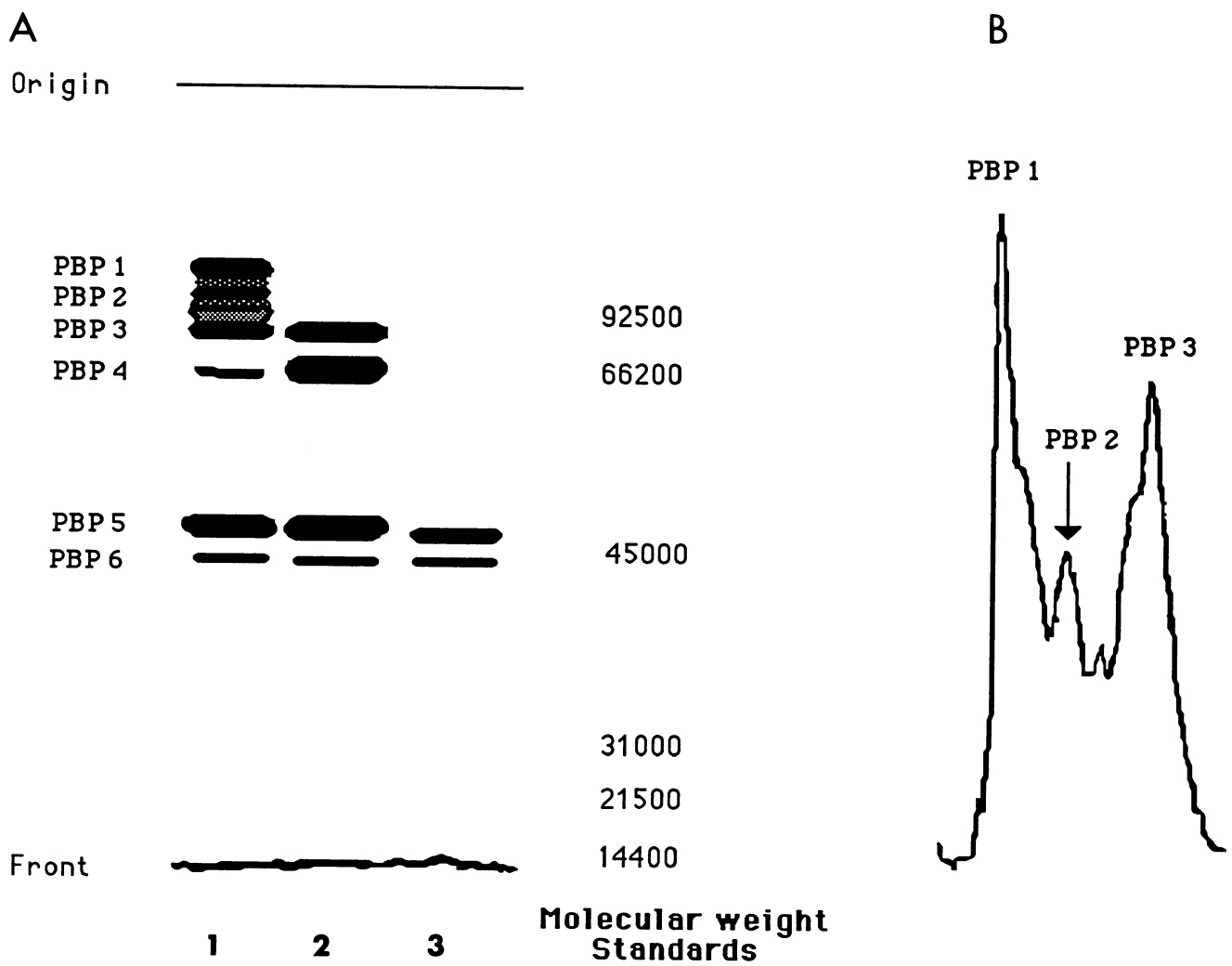

FIG. 1. (A) Digitized image of SDS-PAGE of PBPs in coccal and L-form membranes labeled with [^{3}H]penicillin. Each column represents results from 200 μg of membrane protein. Lanes: 1, streptococcus; 2, osmotically fragile L form; 3, physiologic isotonic L form. The resulting position for each of the molecular weight standards is indicated. Shaded areas correspond to minor components between PBPs 1, 2, and 3 indicated in panel B. (B) Tracing of gel showing degree of resolution of coccal PBPs 1, 2, and 3 by laser densitometry.

exhibited a wide range of molecular weights; the membranes of both L forms were devoid of the highest-molecular-weight proteins in the coccus, i.e., PBPs 1 and 2. Also, while the physiologic isotonic L form contained only two PBPs, both of the low-molecular-weight variety, the osmotically fragile L form had four PBPs. Low-molecular-weight PBPs 5 and 6 were present in the membranes of all three organisms. The relative amounts of total PBPs within the membranes of these three organisms, as determined after saturation with labeled penicillin (with one exception; see above and Fig. 1) were 1.00:1.48:0.32 for the coccus and the osmotically fragile and physiologic isotonic L forms, respectively. Finally, the PBP with the highest molecular weight in each of these membranes possessed the greatest amount of radioactivity.

TABLE 1. PBPs in *S. pyogenes* and derived L-form membranes

PBP	Mol wt[a]	% Penicillin bound by[b]:		
		Coccus	Osmotically fragile L form	Physiologic isotonic L form
1	118,000	29	0	0
2	114,000	16	0	0
3	79,000	20	36	0
4	66,200	6	33	0
5	48,000	25	28	79
6	44,000	4	3	21

[a] Molecular weights were determined after concomitant use of standards (see Materials and Methods).
[b] Determined after exposure to a saturating concentration of ^{3}H-penicillin (0.5 μg) for 15 min.

Albumin was added to all media for the initial growth of these three organisms (Fig. 3A–C). It should be emphasized here that albumin binds penicillin and extensive washes are required for its removal from subsequent membrane preparations (T. J. Dougherty, personal communication). This was confirmed when albumin (M_w, 66,200) within the molecular weight standards mixture became the most radioactive when labeled penicillin was added to this commercial preparation. In addition, albumin comigrated with PBP 4 of the parental coccal and osmotically fragile L-form membranes (Table 1). Additional extensive washing of each of

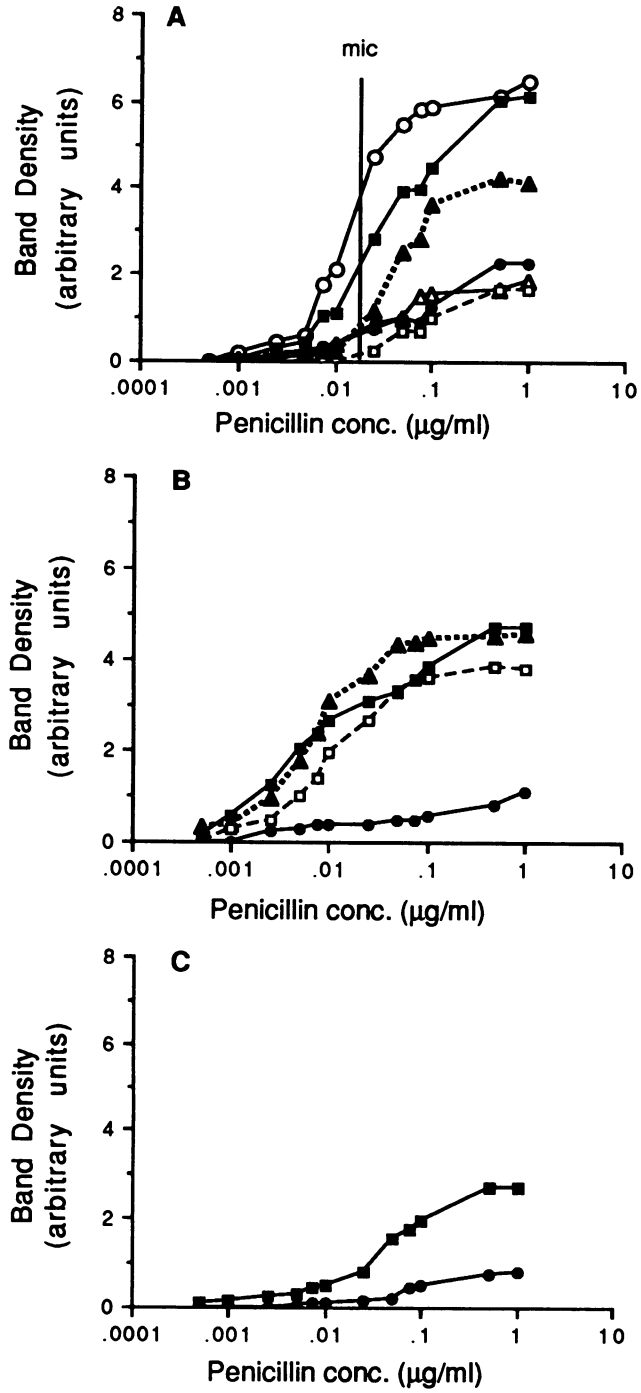

FIG. 2. Titration curves of [^{3}H]penicillin binding by the various PBPs in *S. pyogenes* and derived L-form membranes. A total of 200 μg of membrane protein from each organism was used for each concentration of antibiotic used. (A) Streptococcus; (B) osmotically fragile L form; (C) physiologic isotonic L form. MIC of penicillin is indicated for only the intact coccus. Symbols: ○, PBP 1; Δ, PBP 2; ▲, PBP 3; □, PBP 4; ■, PBP 5; ●, PBP 6.

these three labeled membrane preparations (i.e., 15 mg of total membrane protein, each washed 4, 8, and 10 times, each time with 5 ml of phosphate buffer) failed to show any decrease in radioactivity. Also, although grown with albumin, the physiologic isotonic L-form membrane lacked PBP 4 (Table 1 and Fig. 1). This observation, coupled with the absence of albumin as deduced by lack of radioactivity or positive staining in gels of PBPs from the membrane of the same L form, proves that the method used for obtaining and preparing these three membranes was adequate for the removal of all traces of medium albumin. Therefore, PBP 4 detected in the coccus and its osmotically fragile L form is not residual albumin.

Deacylation of penicilloyl-PBP complexes in labeled membranes. Figure 4A through C illustrates that deacylation of the various PBPs in each of the membranes examined was not linear (for an exception, see below) over the period of examination. The ratio of total band densities at zero time for these different membranes is not the same as has already been indicated above. These data were obtained from different gels, and, as is known, band densities vary from gel to gel. Of particular interest here was the apparent variability in deacylation by the various PBPs within the same membrane as well as by the same PBP compared within each of the different membranes. Differences in the rates (half-life) of deacylation of the various PBPs could not be related (i) to differences in the generation time of the intact organisms, (ii) (in the L forms) to differences in the concentration of NaCl needed for growth, or (iii) to differences in the molecular weights of these PBPs. Of note were the comparative linearity and increased half-life (i.e., slower deacylation) of the four PBPs, as well as an apparent increase in acylation before deacylation for PBPs 3 and 4, in only the osmotically fragile L form (Fig. 4B). The deacylation rates for the two PBPs of the physiologic isotonic L form (Fig. 4C) were markedly different from rates observed when these enzymes were present in the membrane of the osmotically fragile L form and compared with those of the coccus (Fig. 4A). *r* (correlation coefficient) values were 0.87 to 0.97 for the six PBPs of the coccus, 0.82 to 0.94 for the four PBPs of the osmotically fragile L form, and 0.81 and 0.89 for the two PBPs of the physiologic isotonic L form.

As indicated, a perplexing problem was the continued acylation (even after addition of ample cold penicillin), before deacylation of some of the PBPs in only the osmotically fragile (3% [wt/vol] NaCl) membranes. It was suggested that membranes obtained by use of the X-press may lead to predominately "inside-out" (i.e., inverted) membrane vesicles, as opposed to sonication, which results in scrambled vesicles (11–13, 16). Under these conditions, the possibility remained that cold penicillin might be greatly retarded in its penetration of these inside-out vesicles to terminate binding of labeled antibiotic (Dougherty, personal communication). Therefore, these osmotically fragile L-form membrane preparations were also subjected to sonication before addition of labeled and unlabeled penicillin and then subjected to SDS-PAGE. This was accomplished with a sonifier (model LS75; Branson Sonic Power Co., Danbury, Conn.) equipped with a miniprobe (5-A setting) by three successive treatments in ice, each of 4 to 5 s duration. The results obtained with these X-press plus sonication-treated membranes remained the same (i.e., without added sonic treatment, as shown in Fig. 4B, PBPs 3 and 4).

Secretion of PBPs by intact coccal and L-form cells into the growth medium. Initial studies which used growth media with albumin were unsuccessful because of the inability to detect or resolve labeled PBPs electrophoretically within the massive precipitates which resulted from this protein. However, use of prelabeled and washed coccal and L-form cells suspended in medium without albumin (see Materials and Methods) was successful. Because of nutritional differences between the parental coccus and these L forms, the latter do

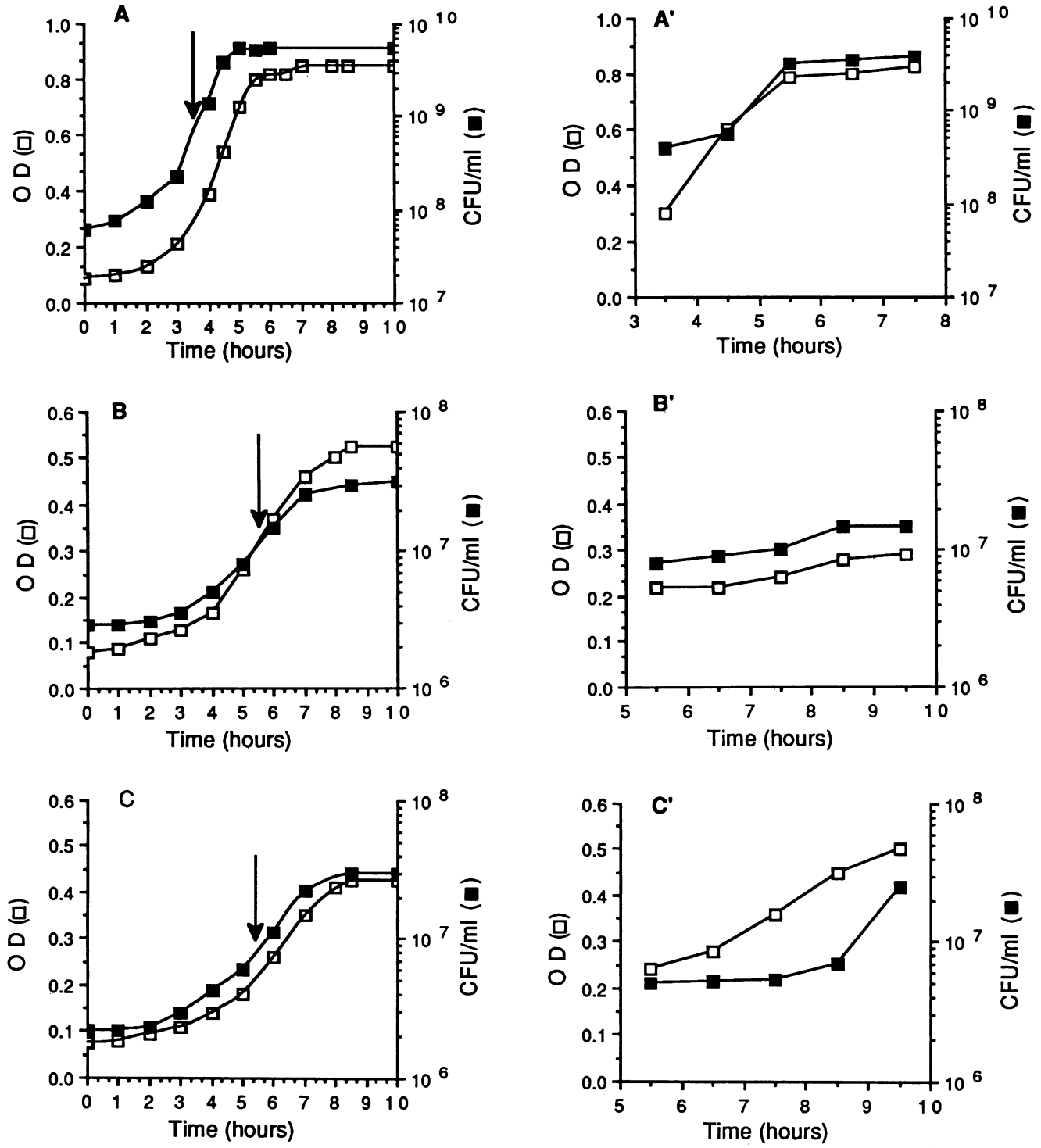

FIG. 3. Growth of *S. pyogenes* and derived L forms in media with (A through C) and without (A′ through C′) albumin. Coccal and L-form cells were labeled with [^{3}H]penicillin for 15 and 45 min, respectively, at the mid-logarithmic phase of growth in medium with albumin (arrow) before being harvested, washed, and suspended in the same volume of prewarmed medium without albumin. (A and A′) Streptococcus; (B and B′) osmotically fragile L form; (C and C′) physiologic isotonic L form.

not grow continuously without albumin in this growth medium. Comparisons in Fig. 3A and A′ show that while medium without albumin did not drastically affect growth (OD and CFU) of the coccus, a significant change was observed with both L forms. For example, growth of the osmotically fragile L form without albumin (Fig. 3B and B′) was affected most, showing only a minimal increase in OD and CFUs over the duration of these experiments. Also, under these same growth conditions the physiologic isotonic L form (Fig. 3C and C′) required approx 2.5 h to initiate cell

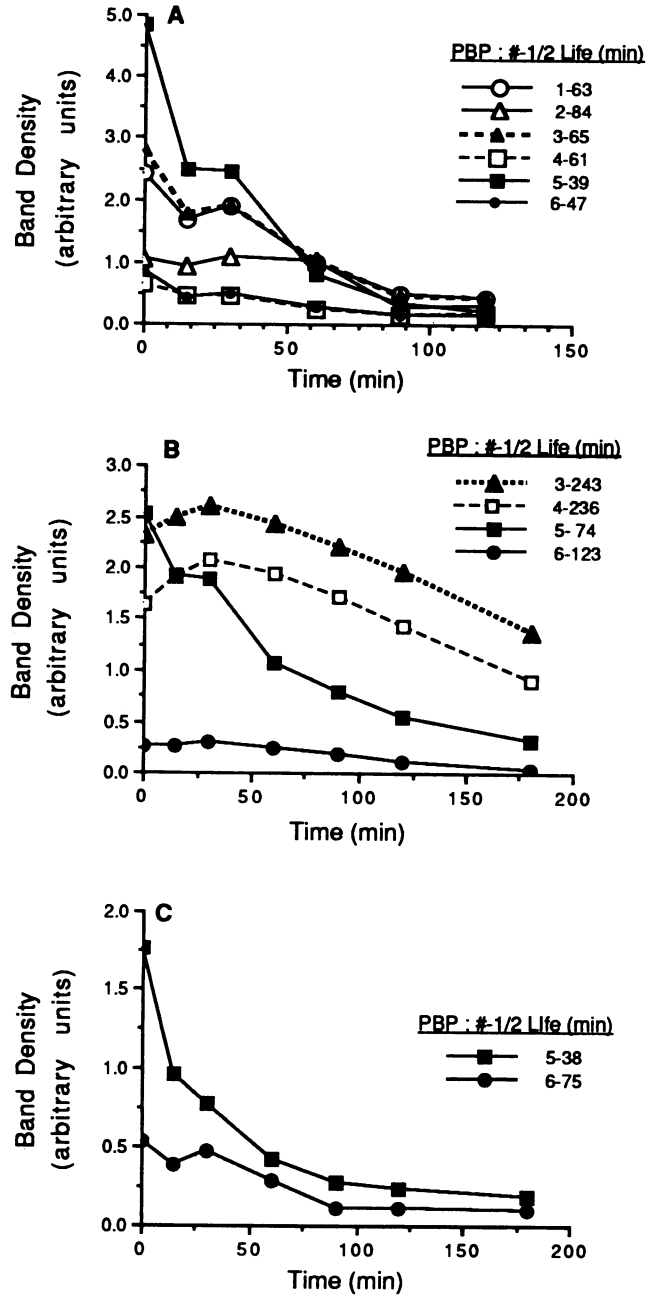

FIG. 4. Deacylation of PBPs. (A) Streptococcus; (B) osmotically fragile L form; (C) physiologic isotonic L form. A total of 200 μg of membrane protein from each organism together with labeled followed by nonlabeled penicillin was used. At times indicated, samples were removed and subjected to SDS-PAGE and fluorography to measure labeled PBPs remaining.

division even though the OD of such cultures was increasing at an almost constant rate during this time period and thereafter. Finally, the OD and CFUs of all cultures with albumin harvested at the mid-logarithmic phase of growth was duplicated when the cells from such cultures were washed and suspended in the same volume of prewarmed medium without albumin (Fig. 3A through C versus A′ through C′). Thus, the washing procedure (see Materials and Methods) used to minimize albumin carry-over during these transfer experiments was not obviously detrimental to these organisms.

Figure 5A through C compares the secretion of PBPs plus a labeled low-molecular-weight material(s) by these intact coccal and L-form cells in medium without albumin in the absence of any added cold penicillin. L-form morphology, as observed by dark-phase light microscopy, remained normal, and there was no decrease in viable count over the course of these experiments (4 h). Corresponding changes in OD and CFUs over this same time are illustrated in Fig. 3A′ through C′ (i.e., zero time in Fig. 5A through C is equivalent to 3.5 and 5.5 h for coccus and L forms, respectively, in Fig. 3A′ through C′). In each case, 100% represents the initial radioactivity within each organism of not less than 1.1×10^5 cpm. The total radioactivity accounted for within the various fractions (Fig. 5) at the conclusion of each experiment was always greater than 96% of that incorporated initially. As is apparent, a greater amount of PBPs was secreted by these L forms than by the parental coccus. However, this secretion by all three organisms ceased after 1 h of incubation. The increase in secretion of labeled PBPs and low-molecular-weight material was accompanied by an equal but opposite decrease in the total cellular radioactivity within these organisms. Gel electrophoresis patterns of the PBPs secreted by these organisms were identical to those from their respective labeled membranes (see above). Likewise, the ratio for the various PBPs being secreted was the same as that within the membrane of each of these organisms. Both L forms secreted more of the labeled, low-molecular-weight material than did the parental coccus (Fig. 5A through C). This material could not be examined by SDS-PAGE because of the much greater concentration of protein in the medium, which resulted in the formation of an insoluble coagulum upon boiling in lysis buffer, and because of an inability to sufficiently dilute this medium without also decreasing its radioactivity below limits detectable by using presensitized X-ray film. Finally, and irrespective of source, this low-molecular-weight material could not be precipitated with trichloroacetic acid (5%, wt/vol).

To determine the nature of this low-molecular-weight material, physiologic isotonic L-form membrane fragments were added to labeled spent acetone-medium supernatants and the mixture was treated as in the penicillin-binding assay. The resulting SDS gels were subjected to radioautography which required at least 1 week for visualization (see Materials and Methods). Only a single weak band, corresponding to that of PBP 5, was always observed. The intensity of this band was considerably less than that for this PBP when membrane preparations were examined. This lesser radioactivity probably accounts for the inability to detect the secretion of the remaining PBP (PBP 6) of this L form; PBP 5 exceeds the amount of PBP 6 in the membrane of this L form by a ratio of nearly 4:1 (Table 1). Finally, addition of labeled penicillin to these reaction mixtures resulted in the same PBP pattern as for the membranes of this L form described earlier (i.e., labeled PBPs 5 and 6 only). The broken beta-lactam ring of penicillin is not regenerated upon deacylation of the penicilloyl-PBP complex (5). Therefore, released penicilloic acid is not capable of reacylation. Likewise, binding of labeled penicillin by bovine serum albumin does not open the beta-lactam ring. These facts and our collective findings indicate that the labeled low-molecular-weight material being released by these intact cells is probably penicilloic acid together with a minute amount of intact, labeled penicillin.

Neither nonspecific binding of [^{3}H]penicillin nor the ap-

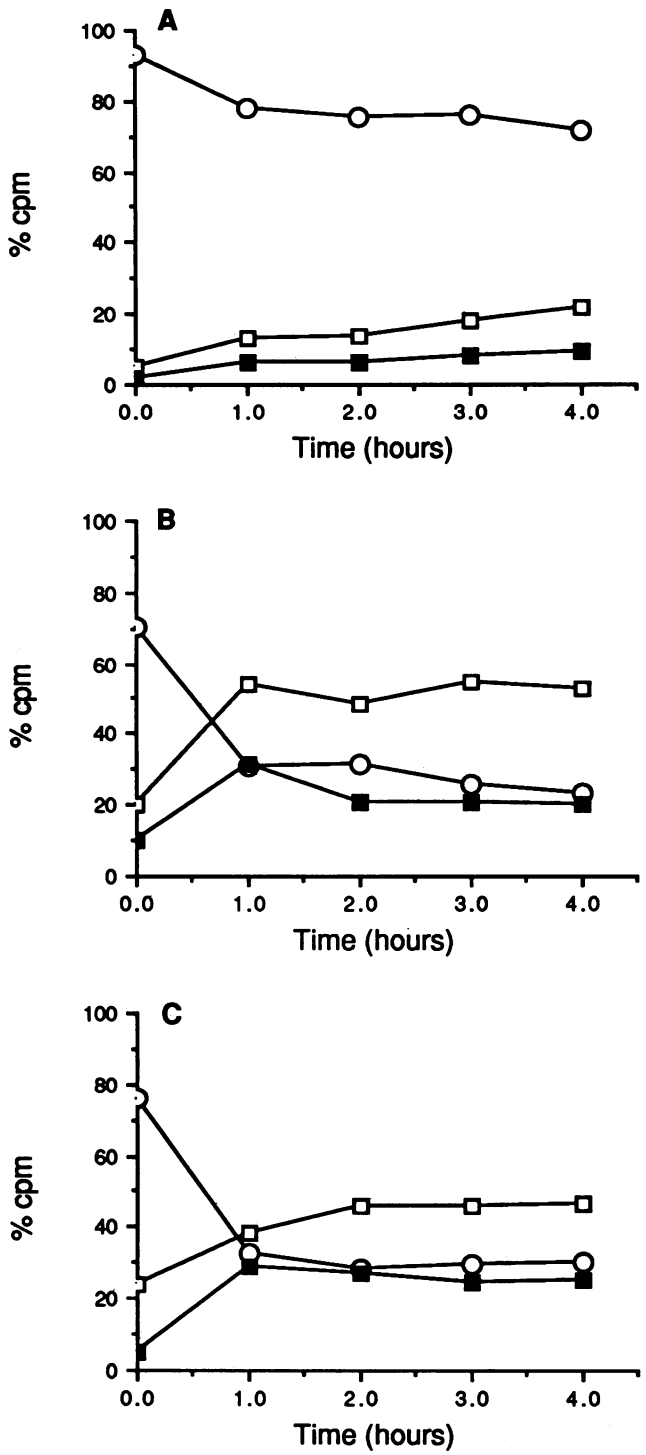

FIG. 5. Secretions with time by intact *S. pyogenes* and derived L-form cells in growth media without albumin. Zero times correspond to penicillin-prelabeled coccal and L-form cells at their respective mid-logarithmic phases of growth in media with albumin and after being washed and suspended in media without albumin (Fig. 3A′ through C′; see also Results). (A) Streptococcus; (B) osmotically fragile L form; (C) physiologic isotonic L form. Symbols: ○, cellular radioactivity; □, secreted low-molecular-weight material(s); ■, secreted PBPs.

pearance of additional radioactive bands with time, indicative of labeled penicillin decomposition, was observed over the course of these studies. The radioactive patterns obtained were highly reproducible, with only PBPs being labelled.

DISCUSSION

These studies show that the number and relative concentrations of the various PBPs within the coccal membrane changed with loss of cell wall synthesis. They also show that these changes are not constant but vary among the membranes of different L forms from the same parental coccus. It had been established previously that these two stabilized (i.e., nonreverting) L forms are completely devoid of all cell wall material. Thus, inhibition of cell wall formation in these L forms is accompanied by a loss of certain PBPs. Whether these PBP changes are representative of inhibition of cell wall formation at a common point or, instead, are an expression of multiple stages of inhibition within a given L-form population was not determined. However, these changes are reminiscent of previous findings after conversion of bacteria to their L forms. It was found that a biochemically heterogenous L-form population may result, with respect to sites of blockage, in the ability of the population to synthesize the rigid cell wall (4, 20). Finally, these results also indicate, as expected, that acylation of all PBPs is not fatal to these two coccal L forms. A similar finding after acylation "of most of the PBP's" was recently reported for a pneumococcus (19).

The data presented here suggest that PBPs 1 and 2 are responsible for, or are involved in, cell wall synthesis in this parental *S. pyogenes*, since they are absent in the two L forms. Thus far, PBPs are known to carry out only two basic reactions that are sensitive to penicillin, transpeptidation and carboxypeptidation (5, 21). The absence of rigid cell wall, but not nucleotide precursor (14), synthesis in the L form probably negates a need for these two high-molecular-weight PBPs. This may, in turn, represent a method of conserving energy needed for the synthesis of now useless proteins by the stabilized L forms. In addition, the absence of two other PBPs (PBPs 3 and 4) in only the physiologic isotonic L form indicates that changes in addition to, or after, adaptation of growth to physiologic isotonic environments have occurred at the subcellular level. Also, the difference in the total number of PBPs (Table 1) between the two L forms is probably not due to a selection process because of differences in growth rates, since the generation time of both L forms is 90 min (see Materials and Methods). The continued presence of low-molecular-weight PBPs 5 and 6 in both L forms is probably vestigial and, although these PBPs are capable of acylation by penicillin, may be functionally insignificant to the L form. However, their presence may become meaningful if they are found to exhibit transpeptidase or carboxypeptidase activity. For example, it would answer the question whether these activities diminish or are totally absent in a resulting physiologic isotonic coccal L form. Bacteria can synthesize the rigid cell wall and grow without the need of a total complement of wall-synthesizing enzymes (1, 22).

In a study of gram-negative organisms and their derived protoplast L forms (no cell wall and stable, i.e., irreversible), it was shown that L forms from two strains of *Proteus mirabilis* lacked a particular PBP (PBP 4, a DD-carboxypeptidase/transpeptidase) compared with the parental organisms (10). It was concluded that this omission might be a reason

for the inability of these L forms to synthesize peptidoglycan and to revert to the normal bacterial state. Similarly, PBP 4 was also lacking in a stable protoplast L form of another proteus, *Proteus vulgaris* (6). By comparison, the mycoplasmas, which are morphologically very similar to bacterial L forms, are devoid of all PBPs (10).

The relative insensitivity (i.e., low affinity) of the low-molecular-weight PBPs in gram-positive organisms to penicillin has been noted (21). However, in the present study, a difference in sensitivity was observed for, presumably, the same PBP (PBP 4) within the coccal and osmotically fragile L-form membranes. Likewise, while a high-molecular-weight PBP (PBP 2) from parental *S. pyogenes* type 12 was relatively insensitive to penicillin in vitro, a possibly related PBP (PBP 2 a+b; average M_w, 81,500) from *S. pyogenes* (T4/56) was most sensitive to this antibiotic (7). Therefore, sensitivity or high affinity to penicillin may be related not only to M_w but also to the membrane environment containing it (see below). In this regard the increased affinity for penicillin of PBP 4 in only the osmotically fragile L form is particularly noteworthy if, as suggested in this paper, it is not involved in cell wall formation in *S. pyogenes* type 12. The molecular weights of the PBPs of this parental coccus were similar, but not identical, to those of another *S. pyogenes* examined earlier (7).

Previously, studies in this laboratory had investigated the lipid intermediates for peptidoglycan synthesis in the osmotically fragile L form included in the present study (15). These enzymatic studies revealed that the membrane of this L form, compared with that of the parental *S. pyogenes*, lacked phosphokinase and transferase activities. Also, it was not possible to conclude whether the specific lesion in this L form existed at the level of enzyme molecules or endogenous lipid substrates. But it was clear that this membrane was unable to form significant levels of lipid intermediates for peptidoglycan synthesis from exogenous nucleotide substrates. As already indicated, PBPs 1 and 2 are probably involved in cell wall synthesis in *S. pyogenes* type 12. Therefore, loss of these PBPs may allow for L-form genesis and probably accounts for the defective synthesis of the lipid intermediates for peptidoglycan synthesis detailed earlier for the osmotically fragile coccal L form (15). In any event, these collective data now establish that lack of cell wall synthesis in these coccal L forms is due, at least in part, to a change at the enzymatic level.

The present study confirms the results of Gutmann et al. (7) that unusually stable complexes of PBPs with penicillin do not occur in *S. pyogenes*. This has now been extended to two of its derived L forms. However, the average rate of deacylation of the PBPs in the membrane of the osmotically fragile L form was considerably slower, by 2.8-fold, than that of the physiologic isotonic L form and parental coccal membranes, suggesting a greater penicilloyl-PBP complex stability in this osmotically fragile L form. Electron resonance spectroscopy analyses of these same membranes had established a very marked difference in their lipid chain rigidity; the order is physiologic isotonic L form > osmotically fragile L form > parental coccus (2). The extreme variance in the half-lives of three of the four PBPs observed within the osmotically fragile L-form membrane versus half-lives of these presumed same PBPs in the parental coccal membrane may be directly related to this difference in membrane lipid chain rigidity. A result of this change may be this longer half-life for most (but not all; e.g., PBP 5) of the penicilloyl-PBP complexes because of the different membrane environment surrounding these particular PBPs. However, the continued acylation, before deacylation, of certain PBPs within the osmotically fragile L-form membrane, even after addition of copious amounts of nonlabeled penicillin, cannot be due to the type of vesicles resulting (i.e., not a penicillin accessibility problem) in these membrane preparations (see Results) and, at present, remains unexplained. It had been established that deacylation rates for PBPs of *S. pyogenes* are comparable when measured in isolated membranes and whole cells (7).

The greater secretion of PBPs by intact cells of the two L forms than that of parental coccal cells was not dependent upon continued cell division or on the addition of exogenous, unlabeled penicillin. The amount of PBPs released by the parental coccus remained minimal even though growth continued. Also, lack of albumin did not increase these secretions by the L forms. The amount of PBPs, penicilloic acid, and minimal free, labeled penicillin released remained constant in the presence or absence of added albumin. Albumin was omitted only to facilitate obtaining successful PBP gel electrophoresis patterns (see Results). As already mentioned, the membrane of the osmotically fragile L form contained a significantly higher content of PBPs than did the membranes of the parental coccus and its physiologic isotonic L form. Therefore, the possibility that the elevated PBP content in this membrane is due to the increased osmolarity needed for growth of this L form cannot be denied, even though no such change resulted when the parental coccus was grown in osmotically fragile L form medium (see Results). But it is unlikely that this need for an increased osmolarity accounts for the two additional PBPs present in the osmotically fragile L form compared with the PBP content of the physiologic isotonic L form. Nevertheless, the amounts of PBPs secreted by both intact L forms remained similar, suggesting a greater retention by the osmotically fragile L-form membrane. The cessation of secretion of PBPs, penicilloic acid, and free penicillin by all three organisms after 1 h was probably due to the absence of added nonlabeled penicillin.

Two consecutive defects in cell wall synthesis have been demonstrated in the derived, stabilized coccal L form, a decrease in the necessary lipid intermediates and, now, the probable loss of the subsequent transpeptidation reaction because of loss of certain required coccal PBPs.

ACKNOWLEDGMENTS

We express our gratitude to A. Tomasz, Rockefeller University, N.Y., for making available to us his laboratory and for his generous supply of labeled penicillin. We also thank T. Dougherty, Bristol-Myers Co., Wallingford, Conn., for his methodologies and discussions which made this study possible.

This investigation was supported by Public Health Service grant AI-11161 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Blumberg, P. M., and J. L. Strominger.** 1971. Inactivation of *D*-alanine carboxypeptidase by penicillins and cephalosporins is not lethal in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **68:** 2814–2817.
2. **Chevion, M., C. Panos, and J. Paxton.** 1976. Membrane studies of *Streptococcus pyogenes* and its L-form growing in hypertonic and physiologically isotonic media. An electron spin resonance spectroscopy approach. Biochim. Biophys. Acta **426:**288–301.
3. **Dougherty, T. J., A. E. Koller, and A. Tomasz.** 1980. Penicillin-binding proteins of penicillin-susceptible and intrinsically resistant *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **18:**730–737.

4. **Fodor, M.** 1965. Studies on the cell wall mucopeptide synthesis of staphylococcal L-forms. Naturwissenschaften **52**:522.
5. **Frere, J.-M.** 1985. Penicillin-sensitive enzymes in peptidoglycan biosynthesis. Crit. Rev. Microbiol. **11**:299–396.
6. **Ghuysen, J.-M., and M. Nguyen-Disteche.** 1986. β-Lactam-induced *Proteus* L-forms, p. 127–162. *In* S. Madoff (ed.), The bacterial L-forms. Marcel Dekker, Inc., New York.
7. **Gutmann, L., R. Williamson, and A. Tomasz.** 1981. Physiological properties of penicillin-binding proteins in group A streptococci. Antimicrob. Agents Chemother. **19**:872–880.
8. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of tritium and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**:335–341.
9. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
10. **Martin, H. H., W. Schlif, and H.-G. Schiefer.** 1980. Differentiation of Mycoplasmatales from bacterial protoplast L-forms by assay for penicillin binding proteins. Arch. Microbiol. **127**:297–299.
11. **Owen, P., and H. R. Kaback.** 1978. Molecular structure of membrane vesicles from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **75**:3148–3152.
12. **Owen, P., and H. R. Kaback.** 1979. Immunochemical analysis of membrane vesicles from *Escherichia coli*. Biochemistry **18**: 1413–1422.
13. **Owen, P., and H. R. Kaback.** 1979. Antigenic architecture of membrane vesicles from *Escherichia coli*. Biochemistry **18**: 1422–1430.
14. **Panos, C.** 1986. Macromolecular synthesis, survival, and cytotoxicity of an L-form of Streptococcus pyogenes, p. 59–97. *In* S. Madoff (ed.), The bacterial L-forms. Marcel Dekker, Inc., New York.
15. **Reusch, V. M., Jr., and C. Panos.** 1976. Defective synthesis of lipid intermediates for peptidoglycan formation in a stabilized L form of *Streptococcus pyogenes*. J. Bacteriol. **126**:300–311.
16. **Rosen, B. P., and J. S. McClees.** 1974. Active transport of calcium in inverted membrane vesicles of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **71**:5042–5046.
17. **Spratt, B. G.** 1977. Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. **72**:341–352.
18. **Tipper, D. J.** 1979. Mode of action of β-lactam antibiotics. Rev. Infect. Dis. **1**:39–53.
19. **Tuomanen, E.** 1986. Newly made enzymes determine ongoing cell wall synthesis and the antibacterial effects of cell wall synthesis inhibitors. J. Bacteriol. **167**:535–543.
20. **Ward, J. B.** 1975. Peptidoglycan synthesis in L-phase variants of *Bacillus licheniformis* and *Bacillus subtilis*. J. Bacteriol. **124**: 668–678.
21. **Waxman, D. J., and J. L. Strominger.** 1983. Penicillin-binding proteins and the mechanism of action of β-lactam antibiotics. Annu. Rev. Biochem. **52**:825–869.
22. **Williamson, R., and A. Tomasz.** 1985. Inhibition of cell wall synthesis and acylation of the penicillin binding proteins during prolonged exposure of growing *Streptococcus pneumoniae* to benzylpenicillin. Eur. J. Biochem. **151**:475–483.