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Effects of Sublethal Concentrations of Benzylpenicillin on *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa produces a low basal level of β-lactamase (0.002 to 0.004 IU/mg of protein when benzylpenicillin is used as substrate). The β-lactamase specific activity can be increased several hundredfold by growing the bacteria in the presence of β-lactam antibiotics. This induction was studied in *Pseudomonas aeruginosa* 1822s. The single-cell resistance to benzylpenicillin was 750 μg/ml. In liquid culture all concentrations of benzylpenicillin tested (25 to 2,000 μg/ml) affected the bacteria similarly: β-lactamase formation was induced, the cells became cholate sensitive, growth rate decreased, filaments were formed, and β-lactamase was excreted. The effect appeared earlier the higher the concentration of the antibiotic. Most of the effects obtained are concerned with the functioning of the outer membrane. The excretion of β-lactamase seems to be due to an opening of the periplasmic volume rather than to lysis of the cells. Carbenicillin gave the same effects as benzylpenicillin at the same concentrations; the 10-fold lower resistance to carbenicillin than to benzylpenicillin can be explained by the inability of the inducible β-lactamase to hydrolyze carbenicillin. The induced β-lactamase was first cell bound and to a great extent located in the periplasmic volume, but later it was excreted into the medium. This extracellular activity was responsible for the detoxification of the medium. This is analogous to the behavior of gram-positive bacteria rather than to that of *Enterobacteriaceae*.

Gram-negative bacteria are generally more resistant to antibiotics than are their gram-positive counterparts. This is particularly true of *Pseudomonas*. Two main factors are responsible for the resistance of gram-negative bacteria to β-lactam antibiotics; these are intrinsic resistance and the production of β-lactamase by the cells (see review by Richmond and Sykes [16].

Intrinsic resistance is due to the presence of a permeability barrier in the outer membrane of gram-negative bacteria (1, 5, 24). This barrier restricts the access of antibiotics to their targets.

β-Lactamases have been extensively studied both in gram-positive (2) and gram-negative bacteria (16). The β-lactamases produced by gram-positive bacteria are extracellular enzymes, many of which are inducible. Among the gram-negative bacteria at least 15 types of β-lactamases have now been identified (16). These enzymes are cell bound and may be either constitutive or inducible. Three types of β-lactamases have been found among strains of *Pseudomonas aeruginosa*. One of these enzyme types seems to be present in all strains of *P. aeruginosa*, namely, the inducible enzyme described by Sabath et al. (6, 20, 21). Maximal induction is obtained using high concentrations of inducer for long periods (4, 6, 21). This makes the induction of β-lactamase a rather unphysiological response, since growth is greatly impaired by these high concentrations of antibiotic inducer. A study of the kinetics of β-lactamase induction is reported in the companion paper (14).

In the present study we have investigated the physiological effects of sublethal concentrations of benzylpenicillin on *P. aeruginosa* strain 1822s. The aim of the study has been to elucidate the significance of the inducible enzyme for the survival of cells and populations of *P. aeruginosa* in the presence of β-lactam antibiotics. Induction of β-lactamase activity was obtained by penicillin concentrations 30 times lower than the single-cell resistance of the organism. A preliminary report has been published elsewhere (13).

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MATERIALS AND METHODS

Materials and methods were as described in the preceding paper (14). The strain used was *P. aeruginosa* 1822s, which is derived from strain 1822 (9) by the loss of the β-lactamase plasmid (25).

RESULTS

Resistance to penicillins. Single-cell resistance of *P. aeruginosa* 1822s against a number of β-lactam antibiotics was very high. However, resistance to carbenicillin was about 10 times lower than that to the other substances (Table 1). The effect of benzylpenicillin on strain 1822s was tested in liquid culture. Growth, measured by optical density (Fig. 1), was affected by concentrations of benzylpenicillin which were far less than the single-cell resistance (see Table 1). However, at 25 μg/ml the effect was obtained only after about 3 h of incubation. At higher concentrations, growth was impaired earlier and the effect was more pronounced (cf. Fig. 6).

Phase-contrast microscopy revealed that 25 μg of benzylpenicillin per ml caused formation of filaments. At higher concentrations of penicillin the filaments had a tendency to stick together in bundles that at 1,000 μg/ml were visible to the naked eye. At the highest concentrations of penicillin tested a great portion of the filaments appeared to be empty. Some swollen cells and cell fragments could also be seen under these conditions. After 5 h the optical density curves accelerated again and this was linked to a rapid increase in the frequency of normal rods.

The decrease in growth rate is also apparent from the protein measurements shown in Fig. 2. At 100 and 200 μg of benzylpenicillin per ml, there was some leakage of protein from the cells after 4 and 3 h, respectively. At 1,000 μg/ml there was a rapid increase in the amount of extracellular protein up to about 50%.

TABLE 1. *The inducible β-lactamase of P. aeruginosa 1822s*

β-Lactam antibiotic	Relative rate of hydrolysis[a]	Single-cell resistance[b] (μg/ml)	Crypticity[c]
Benzylpenicillin	100	750	70
Ampicillin	<3	750	60
Carbenicillin	0	80	55
Cephaloridine	480	750	46

[a] Determined as described by Richmond and Sykes (16).

[b] Determined on LA plates as described by Nordström et al. (12).

[c] Ratio between rate of hydrolysis by disrupted cells and by intact cells (16).

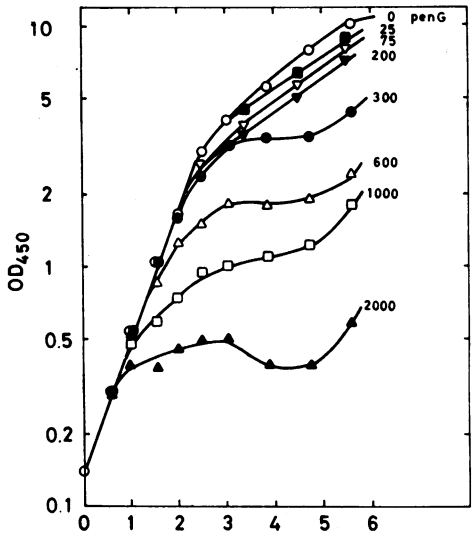

FIG. 1. *Effect of benzylpenicillin on growth of P. aeruginosa 1822s. The bacteria were grown in LB medium for at least 10 generations in the logarithmic phase. At an optical density of about 1 (zero time) the culture was diluted fivefold into prewarmed (37 C) LB medium containing benzylpenicillin, and incubation continued at 37 C. Optical density was measured at intervals. The concentrations of benzylpenicillin tested are given as micrograms per milliliter in the figure.*

Hydrolysis of penicillin by growing cultures. To test the hypothesis that resumption of growth might be due to detoxification of the medium by the inducible β-lactamase, the concentration of penicillin in the culture was measured at different times (Fig. 3). The result was similar at all concentrations of benzylpenicillin tested (range 25 to 2,000 μg/ml)—rapid hydrolysis after a lag period. This lag was shorter the higher the concentration of penicillin (cf. Fig. 6). In all cases the penicillin added had been hydrolyzed after 4 to 4.5 h, the rate of hydrolysis increasing with the penicillin concentration. The time of complete detoxification corresponded well with the time when the optical density curves of Fig. 1 accelerated again. Hence, resumption of growth of the population was due to the disappearance of the antibiotic.

β-lactamase activity after growth in presence of benzylpenicillin. The activity of β-lactamase was measured in 60 clinical isolates of *P. aeruginosa* grown in penicillin-free medium. All strains tested produced a basal level of β-lactamase (0.002 to 0.004 U/mg of protein) (13).

The basal level of β-lactamase found in *P. aeruginosa* 1822s (0.002 U/mg of protein) is not enough to count for the rate of hydrolysis observed in Fig. 3. The slope of the curve for 2,000 μg of benzylpenicillin per ml and the corresponding protein value (Fig. 3) gives an activity exerted by the culture of 0.34 U/mg of protein, which is 200 times the basal activity. This induction was studied at different concentrations of benzylpenicillin (Fig. 4). There was a considerable lag before any increase in β-lactamase activity was detected. This lag was shorter the higher the concentration of penicillin tested (cf. Fig. 6).

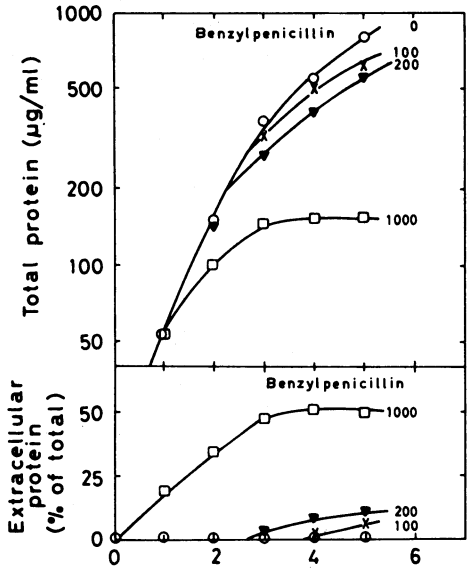

FIG. 2. *Effect of benzylpenicillin on growth of P. aeruginosa 1822s measured as protein. Experimental conditions were as in Fig. 1. (Top) Protein content of the culture; (bottom) extracellular protein as percent of total protein. The concentrations of benzylpenicillin tested are given as micrograms per milliliter in the figure.*

Initially β-lactamase was cell bound, with β-lactamase being excreted into the medium at a later stage (Fig. 4). The excretion was not due to lysis of a great part of the culture, but rather to an opening of the periplasmic volume. Protein appeared in the medium (dotted curve in Fig. 4) at the same time as β-lactamase. However, the relative excretion of protein was only 11%, whereas 55% of the β-lactamase was extracellular after 4.5 h of incubation.

Figure 4 also shows the hydrolysis and optical density curves (top part) and the β-lactamase activity exerted by the growing culture; the latter values were taken from the slopes of the curve showing the hydrolysis of benzylpenicillin. It is evident that the hydrolysis observed in the culture was almost completely due to the extracellular β-lactamase activity. The cell bound β-lactamase thus is not used by the cells to its full capacity. This can be compared to the crypticity values shown in Table 1.

Cholate sensitivity induced by penicillin. The excretion of periplasmic β-lactamase by cells growing in the presence of penicillin sug-

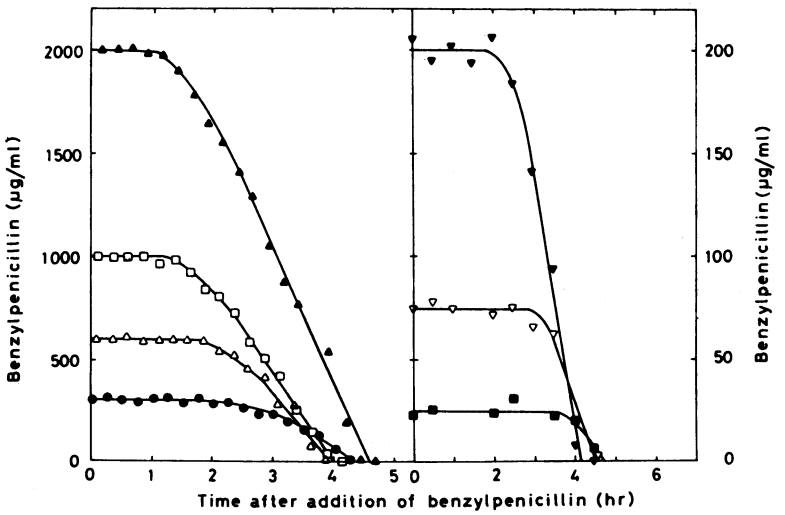

FIG. 3. *Hydrolysis of benzylpenicillin by growing cultures of P. aeruginosa 1822s. Experimental conditions were as in Fig. 1. Penicilloic acid was assayed microiodometrically at intervals and subtracted from the penicillin added initially. The left part of the figure shows the concentration range 0 to 2,000 and the right part of the range 0 to 200 μg of benzylpenicillin per ml.*

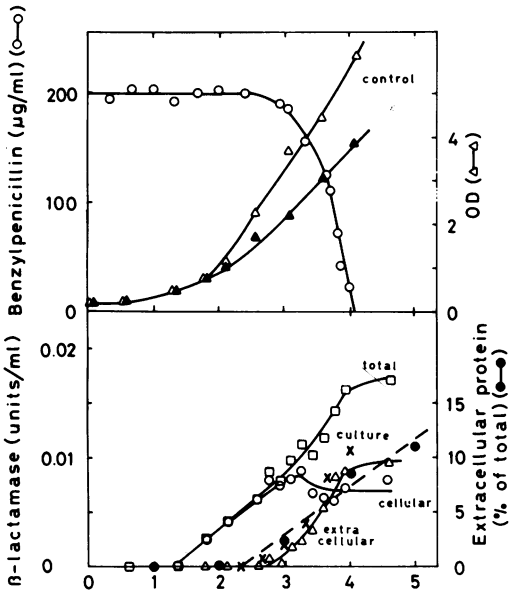

FIG. 4. *Induction of β-lactamase activity in P. aeruginosa 1822s growing in the presence of 200 μg of benzylpenicillin per ml of LB medium. The bacteria were grown for at least 10 generations in the logarithmic growth phase. At an optical density at 450 nm (OD_{450}) of about 1 (zero time) the culture was diluted fivefold into prewarmed (37 C) LB medium containing 200 μg of benzylpenicillin per ml. Samples were taken at intervals and assayed for OD_{450}, benzylpenicillin, β-lactamase, and extracellular protein. The top part of the figure shows the concentration of benzylpenicillin (○) and OD of the culture (▲) and of a control culture that at zero time was diluted fivefold into LB medium without any penicillin (△). The bottom part of the figure shows β-lactamase activity (□, total; ○, cellular; △, extracellular). The rate of penicillin hydrosis exerted by the growing culture was calculated from the slope of the benzylpenicillin curve (x).*

gested that the bacterial envelope had been changed in some way. As reported in a previous paper (1), cells become cholate sensitive by treatment with penicillin. The time after which this sensitivity was manifested was shorter the greater the penicillin concentration (cf. Fig. 6). Cholate sensitivity was also induced by carbenicillin and methicillin.

Comparison of the effect of carbenicillin and benzylpenicillin. The inducible β-lactamase cannot use carbenicillin as substrate and the enzyme is inhibited by this β-lactam drug. Therefore, the effect of carbenicillin on a growing culture was compared with that of benzylpenicillin (Fig. 5). Several hours elapsed before benzylpenicillin hydrolysis started in the culture. Carbenicillin was not significantly hydrolyzed, which is consistent with the substrate profile (Table 1). In the presence of benzylpenicillin, β-lactamase started to increase above the basic level after about 1 h. Enzyme induction by carbenicillin was not studied since this penicillin is not hydrolyzed by the inducible β-lactamase. Growth, measured as optical density at 450 nm, was the same in all cultures for the first few hours. After this period growth rate in the presence of β-lactam drugs decreased compared with the control. The time after which this occurred was shorter the higher the

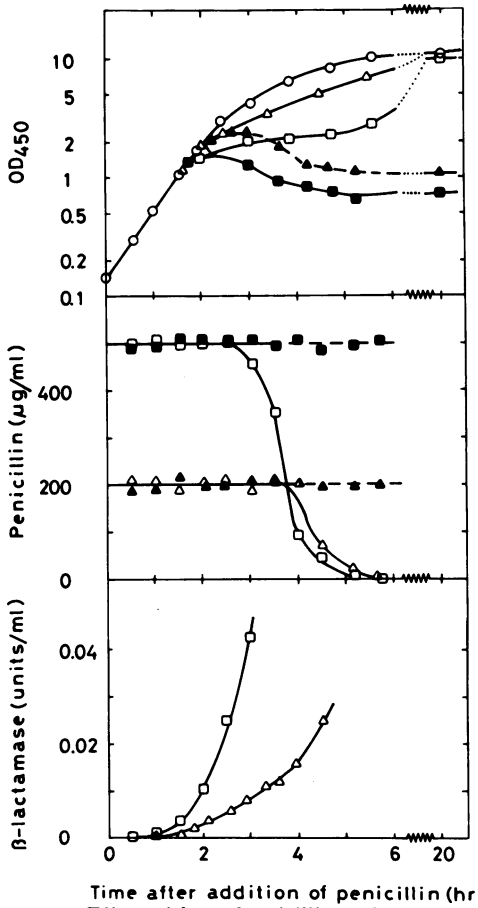

FIG. 5. *Effect of benzylpenicillin and carbencillin on P. aeruginosa 1822s. The bacteria were pregrown in LB medium for at least 10 generations in the logarithmic growth phase. At an optical density at 450 nm (OD_{450}) of about 1 (zero time), the culture was diluted fivefold into prewarmed (37 C) LB medium containing 0 (circles), 100 (triangles), and 200 (squares) μg of benzylpenicillin (open symbols) or carbenicillin (closed symbols) per ml. Samples were taken at intervals and assayed for OD_{450}, penicillin, and β-lactamase.*

concentration of the drug. However, the growth curves deviated from the control curve at the same time in the presence of benzylpenicillin as in the presence of carbenicillin. Later, carbenicillin caused lysis, whereas in the presence of benzylpenicillin there was just a reduction in the growth rate. Both penicillins tested caused the formation of filaments. Normal growth rate was resumed when all benzylpenicillin had disappeared from the medium.

DISCUSSION

Resistance of the individual cell to penicillins. The question arises as to whether the inducible enzyme is of any significance to the resistance of the single cell, since induction of the enzyme shows a long lag. In gram-negative bacteria even a low activity of an inducible enzyme is of significance, since the enzyme cooperates with a penetration barrier (the outer membrane) (1, 5, 24; see also discussion of intrinsic resistance by Richmond and Sykes [16]). This can be shown by a comparison of resistance to carbenicillin and benzylpenicillin. Carbenicillin and benzylpenicillin were equally effective in impairing growth; at the same concentration, the lag before the growth curves deviated from the control was identical (Fig. 5). However, the inducible β-lactamase is inactive against carbenicillin (16) and carbenicillin caused lysis of the whole culture even at 100 μg/ml, whereas the culture survived even in the presence of a 10 times higher concentration of benzylpenicillin. Rosselet (17) has shown that mutants in which the β-lactamase can no longer be induced are much less resistant than the wild type. Hence, it can be concluded that the inducible β-lactamase is of significance for resistance to penicillins and cephalosporins.

Resistance of the population. As is apparent by comparing Fig. 1 and 3, the induced β-lactamase activity is very important for the survival of the population. As soon as the antibiotic is completely hydrolyzed, normal growth resumes again and the filaments that are induced revert to normal rods. The benificial effect of the inducible β-lactamase was first demonstrated by Sabath and Abraham (18). Analogous results have been reported for other organisms by several authors (10, 19). However, the survival of the population in the presence of benzylpenicillin was to a great extent due to extracellular β-lactamase. This is analogous to the behavior of gram-positive bacteria rather than to that of enterobacteria; in the latter case β-lactamases are cell bound (periplasmic) (3, 7, 8, 11, 16).

Induction of β-lactamase due to effects on cell wall biosynthesis? In this paper we have described various effects exerted by benzylpenicillin on *P. aeruginosa*. A number of independent parameters have been used: filament and chain formation, growth rate, excretion of periplasmic proteins, cholate sensitivity, and β-lactamase induction. The results have been summarized in Fig. 6. The higher the concentration of benzylpenicillin the earlier the effects appear. Most of the effects are concerned with the functioning of the cell envelope. Cholate sensitivity seems to be due to an opening of the outer membrane (1). Excretion of periplasmic β-lactamase can also be explained by changes in the properties of the outer parts of the envelope. The excretion of β-lactamase seems to be due to an opening of the periplasmic volume rather than to lysis of the cells (Fig. 4).

The various effects obtained by treatment with benzylpenicillin appear in a defined order at all concentrations tested, indicating that they represent different stages of distortion of the bacterial envelope. This could also mean that the real inducer of β-lactamase biosynthesis is a cell wall precursor or a product formed as a by-product of cell wall biosynthesis after addition of penicillin. In this context it may be

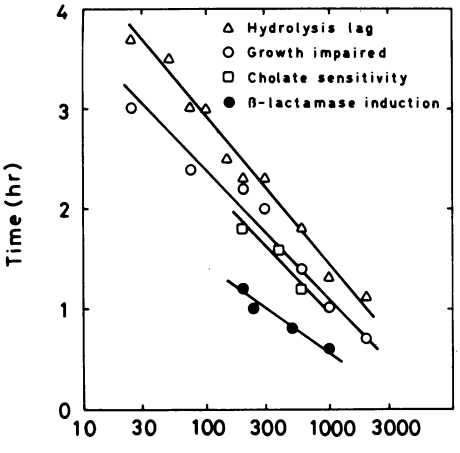

FIG. 6. *Effect of various concentrations of benzylpenicillin on P. aeruginosa 1822s. The lag before hydrolysis of benzylpenicillin started was estimated from Fig. 3, the time before the optical density curves deviated from the control was taken from Fig. 1, the lag before the cells showed a drastically increased sensitivity to sodium cholate was estimated from experiments analogous to that described in Fig. 12 in reference 1, and the lag before β-lactamase production was induced was estimated from Fig. 4 and analogous experiments.*

appropriate to recall that Saz and Lowery (22, 23) have reported that some synthetic peptides can act as inducers and substrates of β-lactamase in staphylococci (22) and *Bacillus cereus* (23). Furthermore, Ozer et al. (15) have reported that a soluble peptidoglycan, also called spore-peptide, is an effective inducer of β-lactamase in *B. cereus*. They conclude that this peptidoglycan is the real inducer even when penicillin is added to a culture of *B. cereus*.

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