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# Pharmacodynamic Effects of Sub-MICs of Benzylpenicillin against *Streptococcus pyogenes* in a Newly Developed In Vitro Kinetic Model

E. LÖWDIN,* I. ODENHOLT, S. BENGTSSON, AND O. CARS

*Antibiotic Research Unit, Department of Infectious Diseases and Clinical Microbiology, University Hospital, Uppsala, Sweden*

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**The pharmacodynamic effects of benzylpenicillin against *Streptococcus pyogenes* were studied in a new in vitro kinetic model in which bacterial outflow was prevented by a filter membrane. Following the administration of an initial dose of antibiotic, decreasing concentrations were produced by dilution of the medium. A magnetic stirrer was placed above the filter to avoid blockage of the membrane and to ensure homogeneous mixing of the culture. Repeated samplings were easily provided through a silicon diaphragm. Streptococci were exposed to a single dose corresponding to 1.5, 10, 100, or 500× the MIC of benzylpenicillin and also to an initial concentration of 10× the MIC of benzylpenicillin, followed by exposure to a repeated dose after 8 h yielding 10 or 1.5× the MIC. Experiments were also performed with 10× the MIC of benzylpenicillin with a half-life of 3 h or an initial half-life of 1.1 h that was altered to 3 h at the time point at which the antibiotic concentrations and MIC intersected. Bacterial killing and regrowth were followed by determining viable counts. The post-MIC effect (PME) was defined as the difference in time for the numbers of CFU in the culture vessel to increase 1 $\log_{10}$ CFU/ml, calculated from the numbers obtained at the time when the antibiotic concentration had declined to the MIC, and the corresponding time for a control culture, grown in a glass tube without antibiotic, to increase 1 $\log_{10}$ CFU/ml. To determine how much of the PME was attributable to subinhibitory concentrations, penicillinase was added to a part of the culture drawn from the flask at the time when the antibiotic concentration had fallen to the MIC. The longest PME was found in the experiments in which the half-life was extended from 1.1 to 3 h at the MIC. This illustrated that sub-MICs are sufficient to prevent regrowth. However, when the half-life was 3 h during the whole experiment, the PME was shorter, indicating that when concentrations decline slowly penicillin-binding proteins will already be present in amounts sufficient for regrowth at the time when the MIC is reached. The PME may prove to be a more reliable factor than the in vitro postantibiotic effect or postantibiotic sub-MIC effect for the design of optimal dosing schedules, since the PME, like the in vivo postantibiotic effect, includes the effects of subinhibitory concentrations and therefore better reflects the clinical situation with fluctuating antibiotic concentrations.**

Following a short antibiotic exposure, many bacteria exhibit a delay before regrowth, the so-called postantibiotic effect (PAE) (3, 6, 15, 29). During the postantibiotic phase (PA phase), various bacteria have been shown to be susceptible to the action of low concentrations (sub-MICs) of the same antibiotic (5). This phenomenon has been named the postantibiotic sub-MIC effect (PA SME) (20). The PA SME is dependent on the antibiotic concentration in the PA phase. For example, in one of our earlier studies in which *Streptococcus pyogenes* was challenged with 0.1× the MIC of benzylpenicillin during the PA phase, the PA SME was 3.3 h, while reexposure to 0.2× the MIC yielded a PA SME of 5.1 h (14). In clinical practice, when intermittent antibiotic dosing is used, antibiotic levels in serum and tissues will gradually decrease and the microorganisms will often be exposed to both supra- and sub-MICs during the dosing interval. The PA SME may thus mimic the in vivo situation better than the PAE, although the experiments are conducted with two static antibiotic levels and therefore do not reflect the true clinical situation. Several in vitro kinetic models have been constructed. These models simulate the serum antibiotic concentration-time curve obtained in humans. There are mainly two different types of models: those that use dilution and those that work by diffusion or dialysis. The dilution models can be made either with or without a filter membrane that prevents the washout of bacteria during the experiments. The aim of the present study was to investigate the pharmacodynamic effects of benzylpenicillin on *S. pyogenes*, especially during the period of the dosing interval when the concentrations were below the MIC. For this purpose a new in vitro kinetic model was developed. Our requirements for the model were that it should be easy to construct, demand little attention during the experiments, and prevent dilution of the bacteria.

* Corresponding author. Mailing address: Department of Infectious Diseases and Clinical Microbiology, University Hospital, S-751 85 Uppsala, Sweden.

## MATERIALS AND METHODS

**Cultures and media.** *S. pyogenes* group A, strain M12, P1800 (National Culture Type Collection), and two clinical isolates of group A beta-hemolytic streptococci (strains 118 and 120), obtained from the Department of Clinical Microbiology, Uppsala University Hospital, Uppsala, Sweden, were used as test strains. Before each experiment the strains were grown in Todd-Hewitt broth in glass tubes and were incubated at 37°C in air with 5% $CO_2$ for 6 h, resulting in approximately $5 \times 10^8$ CFU/ml. In all experiments performed with the model, $CO_2$ was added to the Todd-Hewitt broth by keeping the broth in a box with 5% $CO_2$ in air for at least 2 h before the experiments.

**Antibiotic.** Benzylpenicillin, with known potency, was obtained as a reference powder from Astra Arcus, Södertälje, Sweden. Dilutions were made in distilled water.

**Determination of the MIC.** The MIC was determined by the macrodilution method in Todd-Hewitt broth by using twofold dilutions in 4-ml tubes with an inoculum of approximately $10^5$ CFU of the test strain per ml (8). The tubes were read after 20 h, and the MIC was defined as the lowest concentration of the

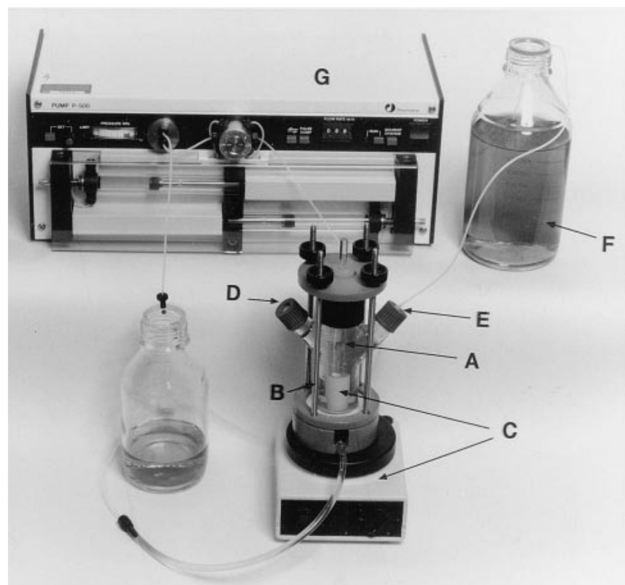


FIG. 1. Design of in vitro model. (A) Culture vessel; (B) construction to clamp the upper and bottom part together; (C) magnetic stirrer; (D) side arm for sampling; (E) side arm supplying fresh medium; (F) vessel containing fresh medium; (G) pump.

antibiotic allowing no visible growth. Determinations of the MIC were performed in triplicate on separate occasions.

**In vitro kinetic model.** An illustration of the kinetic model is shown in Fig. 1. The culture vessel (vessel A; Fig. 1A) consists of a spinner flask (25-ml Bellco spinner flask, suspended bar type, 1967 series; Bellco Glass Inc., Vineland, N.J.). The base was separated from the rest of the flask. A new bottom part with an outlet was constructed by Wiklunds Glas, Stockholm, Sweden. A perforated metal support on which a filter membrane (HAWPO47SO; pore size, 0.45 μm; Millipore) and a prefilter (AP1504700; Millipore) were placed was fitted between the two parts. The parts were tightly clamped together with a special element (Fig. 1B) constructed by the BioMedical Centre, Uppsala, Sweden. In the center of the flask, just above the filter membrane, a magnetic stirrer (Fig. 1C) prevented membrane pore blockage and ensured homogeneous mixing of the culture. In one of the side arms (Fig. 1D), a silicon membrane was inserted to enable repeated sampling. The other arm (Fig. 1E) was connected by a thin plastic tubing to a vessel (Fig. 1F) containing fresh medium. The apparatus was sterilized by autoclaving between every experiment. The model was placed in a thermostatic room at 37°C during the experiments. The medium was drawn from vessel A at a constant rate with a pump (type P-500; Pharmacia Biotech Norden, Sollentuna, Sweden) (Fig. 1G). Fresh sterile medium was sucked into the flask at the same rate by the negative pressure built up inside vessel A. The antibiotic was added through sidearm (Fig. 1D) and was eliminated at a constant rate according to the first-order kinetics $C = C_0 \times e^{-kt}$ (Equation 1), where $C_0$ is the initial antibiotic level in vessel A, $C$ is the antibiotic level at time $t$, $k$ is the rate of elimination, and $t$ is the time that has elapsed since the addition of antibiotic. The constant $k$ is equal to $F/V$, where $F$ is the dilution flow rate and $V$ is the volume of the compartment.

Thus, Equation 1 could be written $C = C_0 \times e\ (-\mathrm{F/V}) \times t$ (Equation 2).

Rearrangement of Equation 2 yields $\ln C = \ln C_0\ (-\mathrm{F/V}) \times t$ (Equation 3).

The flow rate needed to obtain a certain half-life ($t_{1/2}$) could then be calculated, as follows: $F = V \times [(\ln 2 - \ln 1)/t_{1/2})$ (Equation 4), which gives $F = V \times (0.693/t_{1/2})$ (Equation 5).

The volume ($V$) of the part of the culture flask containing bacteria was 108 ml (136 ml when the part below the filter membrane is included).

**Determination of antibiotic concentration.** The concentration of benzylpenicillin was determined every 1 to 3 h during the experiments. A microbiological agar diffusion method was used, with *Bacillus stearothermophilus* ATCC 3032 used as the test strain (4). A standardized inoculum of the spore suspension was mixed with tryptone-glucose agar adjusted to pH 7.4, medium was poured into the plates, and 0.03-ml portions of the samples and standards were applied into the agar wells. All assays were performed in triplicate. The lowest detectable concentration of benzylpenicillin was 0.01 mg/liter.

**Definition of PAE.** The PAE was defined as the difference in time for the bacteria transferred to glass tubes with penicillinase, at the time when the concentration had fallen to the MIC (see below), to increase 1 $\log_{10}$ CFU/ml and the corresponding time for a control culture, grown in a test tube without antibiotic, to increase 1 $\log_{10}$ CFU/ml.

**Definition of PME.** The post-MIC effect (PME) was defined as the difference in time for the numbers of CFU in the culture vessel to increase 1 $\log_{10}$ CFU/ml, calculated from the numbers obtained at the time when the antibiotic concentration had declined to the MIC, and the corresponding time for a control culture, grown in a test tube without antibiotic, to increase 1 $\log_{10}$ CFU/ml.

**Control growth.** To study whether there was a change in the growth rate of the streptococci due to the continuous supply of fresh medium during the experiments, *S. pyogenes* group A, M12, P1800, was grown in the kinetic model without antibiotic. Fresh medium was supplied at a flow rate of 136 ml/h, and the growth rate was compared with that of a control grown in a test tube. Experiments were also conducted with dilution rates of 68 and 272 ml/h without the filter to study the effect of bacterial dilution on the apparent growth rate in the model. All experiments were performed in triplicate.

**Determination of antibacterial activity.** Vessel A was filled with sterile Todd-Hewitt broth, and 1 ml of the test strain culture was added, resulting in approximately $5 \times 10^6$ CFU/ml. Thereafter, different volumes of a solution of 100 g of benzylpenicillin per liter were added to the vessel in order to achieve the desired initial concentration. Repeated sampling for antibiotic concentration and bacterial density was done every 1 to 3 h during the experiments. Dilutions of the culture were performed in sterile phosphate-buffered saline (pH 7.2). Samples of the dilutions (0.1 or 0.01 ml) were plated onto blood-agar plates, the plates were incubated for 24 h in 5% $CO_2$ in air at 37°C, and the numbers of CFU were counted. The results of the estimation of viable counts were based on plates with 10 to 500 colonies per plate. Samples from the waste were also drawn and cultured every 1 to 3 h during the experiments to ensure that there was no filter leakage.

**Bacterial killing and regrowth after a single dose with and without penicillinase.** *S. pyogenes* group A, M12, P1800, was exposed to an initial concentration of 1.5, 10, 100, and 500× the MIC (corresponding to concentrations of 0.023, 0.156, 1.56, and 7.8 mg/liter, respectively), with the $t_{1/2}$ of benzylpenicillin being 1.1 h. Bacterial killing and regrowth were followed, and the PAE and PME were determined as described above. To elucidate the relative importance of exposure time to supra- and sub-MICs for the length of the PME, the streptococci were also exposed to 10× the MIC of benzylpenicillin with a $t_{1/2}$ of 3 h, and additional experiments were performed in which the initial $t_{1/2}$ of 1.1 h was changed to 3 h at the time when the MIC was reached. In all experiments 4 ml of the culture was drawn from the flask at the time when the concentration had fallen to the MIC and was transferred to a glass tube. Eighty microliters of 1,000 U of penicillinase (Sigma Chemical Company, St. Louis, Mo.) per ml was added to the tube to inactivate the benzylpenicillin. The tube was incubated at 37°C, and samples were obtained every 1 to 3 h for bacterial counting. All experiments were performed in triplicate. The clinical isolates were likewise exposed to 10× the MIC of benzylpenicillin with $t_{1/2}$s of 1.1 and 3 h.

**Bacterial killing and regrowth after repeated dosing.** To study the pharmacodynamic effects of a second dose, *S. pyogenes* group A, M12, P1800, was exposed to an initial concentration of 10× the MIC of benzylpenicillin. A $t_{1/2}$ of 1.1 h was applied to the model, and a second dose, yielding 10 or 1.5× the MIC, was given at 8 h (three experiments were performed for each MIC).

## RESULTS

**MICs.** The MICs for *S. pyogenes* group A, M12, P1800, and the clinical isolates 118 and 120 were 0.0156 mg/liter.

**Antibiotic concentration.** The concentrations of benzylpenicillin obtained in vessel A were predictable and showed little variation between experiments (Fig. 2).

**Control growth.** There was no difference in the growth rate in the kinetic model with a filter and a flow rate of 136 ml/h compared with the growth rate of a control growing in a test tube. In experiments without a filter and a flow rate of 68 ml/h, the apparent growth rate was slower than that in experiments with a filter. The impact of bacterial loss was even more pronounced at a flow rate of 272 ml/h, at which the numbers of CFU were constant during the experiments. Thus, at this dilution rate the bacterial doubling time was equal to the rate of reduction of bacteria due to washout. This was calculated to be 21 min (Equation 5), closely corresponding to the doubling time seen in the control tubes, which was 23 min. This finding also indicates that there was no buildup of nondilutable, adherent bacterial populations, as described by Haag et al. (13).

**Bacterial killing and regrowth after a single dose with and without penicillinase.** When *S. pyogenes* group A, M12, P1800, was exposed to an initial concentration of 1.5, 10, 100, or 500× the MIC of benzylpenicillin with a $t_{1/2}$ of 1.1 h, the killing rates in the first 2 h in the model were 0.4 (0.3 to 0.4), 0.8 (0.7 to 0.8), 0.6 (0.5 to 0.7), and 0.7 (0.6 to 0.7) log CFU/ml/h, respectively (values in parentheses are ranges). The corresponding PMEs

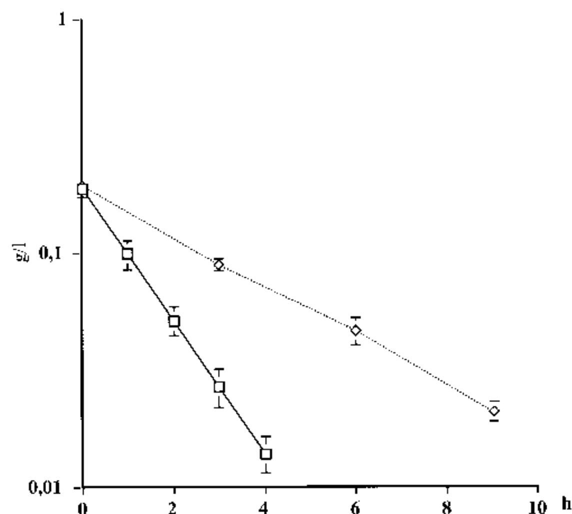


FIG. 2. Concentration curves from the experiments with an initial concentration of 10× the MIC of benzylpenicillin and a $t_{1/2}$ of 1.1 h (□; mean ± standard deviation of 12 experiments) and a $t_{1/2}$ of 3 h (◇; mean ± standard deviation of 3 experiments).

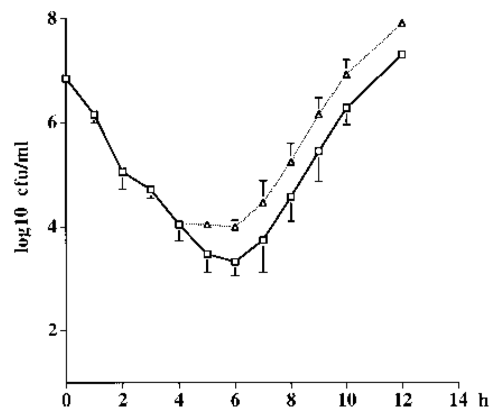


FIG. 3. Killing and regrowth of *S. pyogenes* exposed to 10× the MIC of benzylpenicillin with a $t_{1/2}$ of 1.1 h, with (△) and without (□) the addition of penicillinase (values are means ± standard deviations).

were 2.2, 4.0, 4.0, and 3.8 h. In the experiments with an initial concentration of 10, 100, and 500× the MIC and a $t_{1/2}$ of 1.1 h, the PAE was also measured, and the PAE was always shorter than the PME (Table 1; Fig. 3). When the dilution rate was altered to yield a $t_{1/2}$ of 3 h at the time when the concentration had fallen to the MIC, the PME was 6.8 h and the PAE 3.0 h (Fig. 4). If the $t_{1/2}$ was, instead, 3 h for the whole experiment, both the PAE and the PME were short: 0.2 and 1.2 h, respectively (Fig. 5). The PMEs for clinical isolates 118 and 120 exposed to 10× the MIC of benzylpenicillin with a $t_{1/2}$ of 1.1 h were 3.6 and 1.9 h, respectively, and with a $t_{1/2}$ of 3 h, the PMEs were 1.8 and 1.9 h, respectively. The PAEs and PMEs for *S. pyogenes* group A, M12, P1800, are given in Table 1.

**Bacterial killing and regrowth after repeated dosing.** When a second dose yielding either 1.5 or 10× the MIC of benzylpenicillin was given 8 h after an initial exposure to 10× the MIC, the killing rate in the first 2 h was 0.9 (range, 0.7 to 1.1) and 0.6 (range, 0.4 to 0.7) log CFU/ml/h, respectively, even though the concentration of the lower dose rapidly decreased to sub-MICs. The killing, however, continued longer and regrowth occurred later with the higher dose (Fig. 6A and B).

## DISCUSSION

In earlier studies we have shown that low antibiotic levels (sub-MICs) may have pronounced effects on bacteria in the PA phase, the so-called PA SME (5, 14, 19–21). Many of the animal models that have been developed for pharmacodynamic studies have not distinguished the effect of subinhibitory antibiotic concentrations from the PAE (6, 10, 18, 25, 28). When Täuber et al. (25) investigated the PAE of ampicillin against pneumococci in a rabbit meningitis model, a PAE ranging from 2.5 to 18 h was demonstrated. However, when β-lactamase was injected intracisternally after 2 or 4.5 h, the PAE could no longer be detected, which indicates that the effect first found in this model could instead be attributed to low residual amounts of drug. Craig and colleagues (28) have been able to demonstrate an in vivo PAE for many bacteria-antibiotic combinations using a thigh infection model in neutropenic mice. In those experiments, the PAE was measured from the time that the drug concentration declined to the MIC, and the results thus include the possible additional effects of residual drug at sub-MICs. Since antibiotic elimination is very rapid in mice, the effects of such residual drug concentrations are probably of little importance to the results. If, however, fractionated doses are given to mice (7) or if renal impairment (10) is induced to prolong the $t_{1/2}$s of the drug in order to simulate the profile in human serum, the effects of sub-MICs are more pronounced and a substantial prolongation of the in vivo PAE is found.

One of the advantages with in vitro kinetic models is that human pharmacokinetics can be more easily simulated than they can in animal models. Also, the ethical aspects of experiments with animals are of great concern. A variety of different in vitro models have been developed. One of the first, and the one mostly used, is the one-compartment open model described by Grasso et al. (11). In that model, as in many other-sthe bacterial culture is diluted together with the drug. Mu,

TABLE 1. PAE and PME for *S. pyogenes* group A, M12, P1800, exposed to different concentrations and $t_{1/2}$s of benzylpenicillin[a]

| $t_{1/2}$ and benzylpenicillin MIC | PAE (h) | PME (h) | Time above the MIC (h) |
|---|---|---|---|
| $t_{1/2}$ = 1.1 h | | | |
| 1.5× | ND | 2.2 (1.8–2.5) | 0.4 (0.2–0.5) |
| 10× | 2.8 (2.5–3.5) | 4.0 (3.2–4.9) | 3.6 (3.3–4.0) |
| 100× | 3.0 (2.6–3.5) | 4.0 (4.0–4.1) | 7.3 (7.2–7.3) |
| 500× | 2.1 (0.8–2.6) | 3.8 (1.6–5.2) | 9.4 (9.2–9.5) |
| $t_{1/2}$ = 3.0 h, 10× | 0.2 (0.1–0.3) | 1.2 (0.9–1.8) | 10.2 (10.0–10.5) |
| $t_{1/2}$ = 1.1 h but changed at time of the MIC to $t_{1/2}$ = 3.0 h, 10× | 3.0 (2.7–3.3) | 6.8 (5.7–7.5) | 3.7 (3.5–4.0) |

[a] Values are means of three experiments (ranges). ND, not determined.

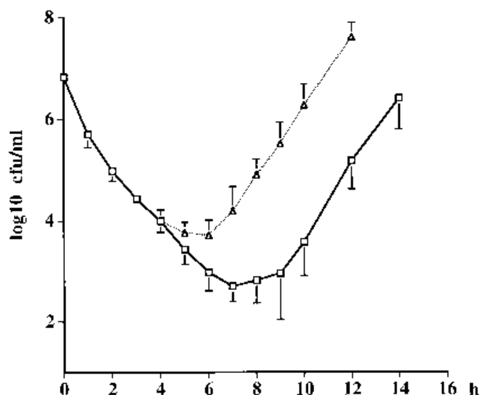


FIG. 4. Killing and regrowth of *S. pyogenes* exposed to 10× the MIC of benzylpenicillin, with the initial $t_{1/2}$ of 1.1 h changed to 3 h at the time of the MIC, with (△) and without (□) the addition of penicillinase (values are means ± standard deviations).

rakawa et al. (16) proposed a mathematical adjustment of the dilution effect, and several investigators have used filter membranes to prevent the washout of bacteria (1, 17, 23, 24, 26). In such models, however, membrane pore blockage could be a problem (1), which becomes more pronounced when antibiotics with short $t_{1/2}$s are studied, since the flow rate across the filter is higher. A way to overcome this problem has been to use diffusion or dialysis of the drug to create the desired antibiotic concentration profile in a separate bacterial compartment (2, 9, 22, 26, 31). Some of these models have been very complex and are thus difficult to sterilize between experiments (22).

In the present report we have described an in vitro model that is easily constructed, fairly inexpensive, and labor effective and that prevents dilution of the bacteria without membrane pore blockage even when short $t_{1/2}$s of the drug are simulated. Furthermore, no buildup of nondilutable, adherent bacterial populations, as described by Haag et al. (13), was demonstrated. We have used the model for further investigation of the pharmacodynamic effects of benzylpenicillin on *S. pyogenes*, especially during the period of the dosing interval, when the concentrations were below the MIC. Haag (12) proposed the term PME for the time to bacterial regrowth after antibiotic concentrations had fallen below the MIC, but he did not include a quantification in his definition of this concept. In the present study, the PME was defined as the difference in time for the bacterial counts in the model to increase 1 $\log_{10}$ CFU

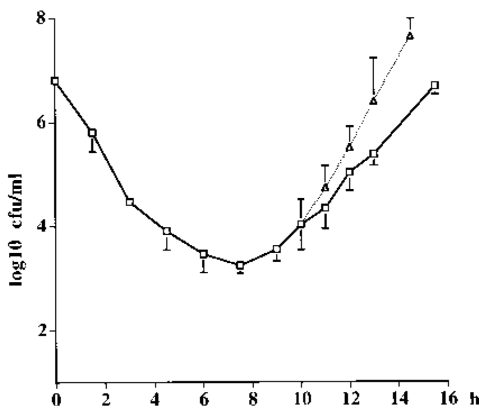


FIG. 5. Killing and regrowth of *S. pyogenes* exposed to 10× the MIC of benzylpenicillin with a $t_{1/2}$ of 3 h, with (△) and without (□) the addition of penicillinase (values are means ± standard deviations).

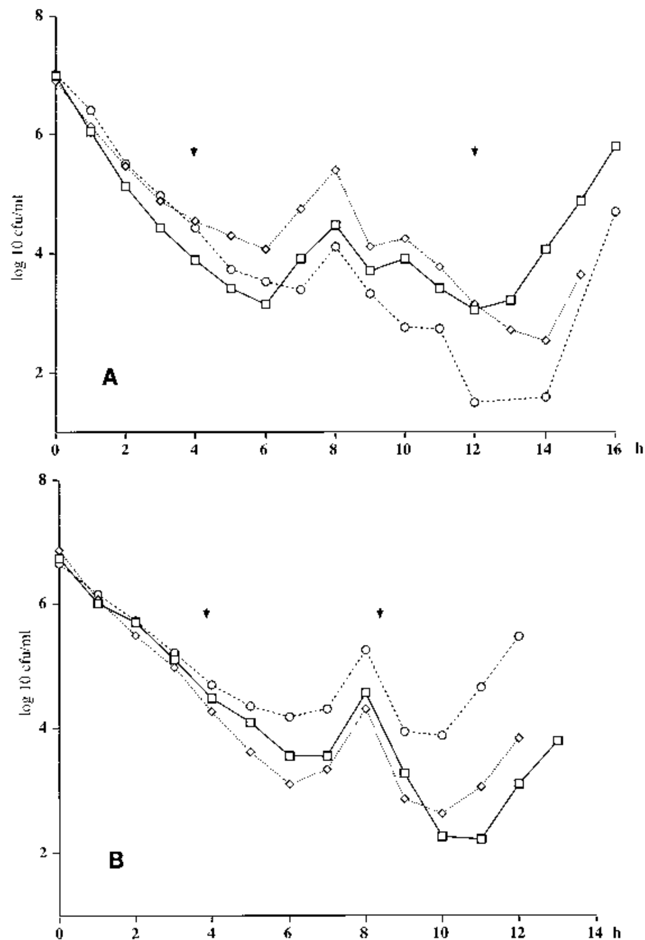


FIG. 6. Exposure of *S. pyogenes* to 10× the MIC of benzylpenicillin with a $t_{1/2}$ of 1.1 h, with an additional dose of 10× the MIC (A) or 1.5× the MIC (B) at 8 h (three experiments each are represented by the three different symbols). Arrows indicate the time when the MIC was reached.

from the numbers obtained at the time when the antibiotic concentration had declined to the MIC and the corresponding time for a control culture not exposed to antibiotic to increase 1 $\log_{10}$ CFU.

In the experiments in which the $t_{1/2}$ was short, the reduction in the numbers of streptococci continued even when the concentration declined to below the MIC, indicating that low drug concentrations are sufficient to maintain the bactericidal mechanisms for a certain period of time. This is in accordance with our earlier findings with static conditions, in which streptococci that had already been treated with benzylpenicillin seemed more vulnerable when they were again exposed to low concentrations of the drug (14, 19). Tuomanen (27) also found that penicillin-pretreated cells were hypersusceptible to the effects of sub-MICs of penicillin compared with the susceptibilities of control bacteria not previously exposed to the antibiotic. Treatment of a part of the culture with penicillinase made it possible to distinguish between the effects of supra-MICs versus sub-MICs. When penicillinase was added at the time point when the concentrations intersected the MIC (PAE), the killing stopped and regrowth occurred earlier. The β-lactams are known to bind covalently to penicillin-binding proteins (PBPs). Tuomanen (27) examined the resumption of cell wall synthesis and the appearance of functional PBPs in pneumococci and *Escherichia coli* after exposure to penicillin. Their results indi-

cated that only a minor fraction of the total content of PBPs is sufficient for a normal rate of cell wall synthesis. In a recent study Yan et al. (30) suggested that the increase in the numbers of PBPs in beta-hemolytic streptococci after the removal of penicillin is due to newly synthesized PBPs and not to deacylation. Yan et al. (30) drew the conclusion that the newly synthesized PBPs are necessary for regrowth during the PA phase.

On the basis of these observations and our experimental results, we propose the following hypothesis. After challenge with an initial suprainhibitory concentration, a certain proportion of PBPs will be occupied by the drug. If the antibiotic is rapidly and completely removed from the culture medium, there is often a delay in regrowth (PAE) which probably corresponds to the time that it takes for the bacteria to synthesize a critical number of PBPs to resume cell division. Also in our kinetic experiments, when a short $t_{1/2}$ was used, there was a delay in regrowth (PME) which started later than the time point when the antibiotic concentrations and the MIC intersected (Fig. 3). The longest PME in our experiments was found when the $t_{1/2}$ of benzylpenicillin was first 1.1 h and was then extended to 3 h at the time point when the concentration had declined to the MIC. This illustrated that sub-MICs seem to efficiently inhibit the newly produced PBPs. However, if the $t_{1/2}$ was 3 h for the whole experiment, the bacteria already started to multiply at the time when the MIC was reached. In these experiments the concentration declines more slowly, remaining close to the MIC for a longer period of time, during which newly produced PBPs may not be fully saturated. The critical number of free PBPs necessary for cell multiplication could therefore be reached earlier and subsequent sub-MICs would not have a substantial effect in preventing regrowth. The MIC is an approximate value and may differ by 1 to 2 dilution steps between measurements. This could explain why in some of the experiments with the long $t_{1/2}$ of the drug, regrowth started even before the MIC used in the experiments was reached. The PME may be one of several possible explanations for the success of intermittent dosing for the treatment of gram-positive infections with β-lactam antibiotics. Our results suggest that although a PAE or PA SME is found in vitro, both of these factors may be less important when the drug has a long $t_{1/2}$ (shorter PME). The PME may thus prove to be a more reliable factor than the PAE or PA SME for the design of optimal antibiotic dosing schedules, because it reflects the clinical situation with fluctuating antibiotic concentrations. In our study, above a concentration of 10× the MIC, the PME was not dependent on either the concentration of benzylpenicillin or the time above the MIC. However, further experiments with different antibiotics and bacteria are necessary in order to establish the presence and significance of this pharmacodynamic parameter.

## ACKNOWLEDGMENTS

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