

## A New Method To Determine Postantibiotic Effect and Effects of Subinhibitory Antibiotic Concentrations

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It has been shown that bacteria in a postantibiotic (PA) phase exposed to subinhibitory concentrations (sub-MICs) of antibiotics show a long delay before regrowth. This effect has been named the PA sub-MIC effect (PA SME). In the present study, we have used a new method to demonstrate this phenomenon. A computerized incubator for bacteria, Bioscreen C (Lab Systems, Helsinki, Finland), which incubates the bacteria, measures growth continuously by vertical photometry, processes the data, and provides a printout of the results was used. With this method, one may easily test several antibiotics against different bacteria for PA effects (PAEs), PA SMEs, and SMEs. In this study, the effects of benzylpenicillin against  $\beta$ -hemolytic streptococci and pneumococci were examined. The bacteria were exposed to 2, 10, or 50 $\times$  MIC for 2 h, washed and diluted, incubated in the Bioscreen C incubator, and then exposed to 0.1 to 0.9 $\times$  MIC. The regrowth was monitored for 20 h. The PAE was calculated as the difference in the time required for the exposed and unexposed bacteria to grow to a defined point ( $A_{50}$ ) on the absorbance curve.  $A_{50}$  was defined as 50% of the maximum absorbance for the control cultures. The PA SMEs were calculated as the difference in the time required for the reexposed cultures and the unexposed controls to reach  $A_{50}$ . The PAEs ranged between 0.6 and 3.2 h and varied little with the concentration used for the induction of the PAEs. At 0.2 $\times$  MIC, the PA SMEs were 2 to 3 h longer than the PAEs. Higher sub-MICs increased this delay before regrowth. Most cultures exposed to sub-MICs alone were only slightly affected compared with the controls.

Earlier studies and clinical experience have shown that in the treatment of streptococcal and pneumococcal infections, intermittent doses of penicillin are successful even if concentrations in serum and tissues fall below the MIC for the bacteria for long intervals (11, 24). These results may partly be explained by the so-called postantibiotic (PA) effect (PAE), i.e., the suppression of bacterial growth that persists after limited exposure to an antibiotic (3). When intermittent dosing is applied in clinical practice, however, there is a gradual decrease in the antibiotic concentration in which suprainhibitory concentrations will often be followed by a period of subinhibitory concentrations (sub-MICs). The effects of these sub-MIC levels on bacteria may be an additional explanation for the success of intermittent dosage schedules. We have shown earlier that in certain antibiotic-bacterium combinations, when a PAE is found, there is a long delay before regrowth when the bacteria are reexposed to sub-MICs during the PA phase (PA sub-MIC effect [PA SME]). The PA SMEs have generally been found to be more pronounced than the direct effect of sub-MICs on bacteria not previously exposed to antibiotics (13-15). In these experiments, we used experimental and control cultures in broth from which serial subcultures were transferred onto solid media, and the numbers of CFUs were counted. However, this technique is time-consuming and laborious. The purpose of the present study was therefore to evaluate a different method for the study of PAE and PA SME. A computerized incubator for bacteria (Bioscreen C; Lab Systems, Helsinki, Finland) which measures growth continuously by vertical photometry (optical density) was used. This model permits an easy screening of different bacteria and antibiotics for PAE and the effects of subinhibitory concentrations.

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### MATERIALS AND METHODS

**Cultures and media.** Two reference strains, *Streptococcus pneumoniae* ATCC 6306, *Streptococcus pyogenes* group A M12 NTCC P1800, and four clinical isolates of group A  $\beta$ -hemolytic streptococci, obtained from the Department of Clinical Microbiology, Uppsala University Hospital, Uppsala, Sweden, (one experiment each) were used as test strains. The cultures were grown in Todd-Hewitt broth for 6 h at 37°C in 5% CO<sub>2</sub> in air, resulting in approximately  $5 \times 10^8$  CFU/ml. Dilutions of the bacteria were made in sterile phosphate-buffered saline, pH 7.2. In all experiments performed in the Bioscreen C incubator, CO<sub>2</sub> was added to the medium by keeping the medium in a box with 5% CO<sub>2</sub> in air for at least 2 h.

**Antibiotic.** Benzylpenicillin was obtained as a reference powder from Astra Research Centre, Södertälje, Sweden.

**Experiments performed with Bioscreen C.** (i) **Apparatus.** A computerized incubator (Bioscreen C [Lab Systems]) providing automatic serial dilutions of bacteria and antibiotics was used in the experiments. It also incubates the bacteria, measures growth continuously by vertical photometry (optical density), using a wavelength of 540 nm, processes the data, and provides a printout of the results.

(ii) **Determination of MIC.** The MICs of benzylpenicillin for the test strains were determined with the Bioscreen C incubator. Twofold serial dilutions of penicillin in Todd-Hewitt broth were made in microplates containing 400- $\mu$ l wells, and the test strains were added to give a bacterial density of approximately  $2 \times 10^5$  CFU/ml. The plates were then incubated in the Bioscreen C. The MIC was defined as the lowest concentration of the antibiotic that prevented

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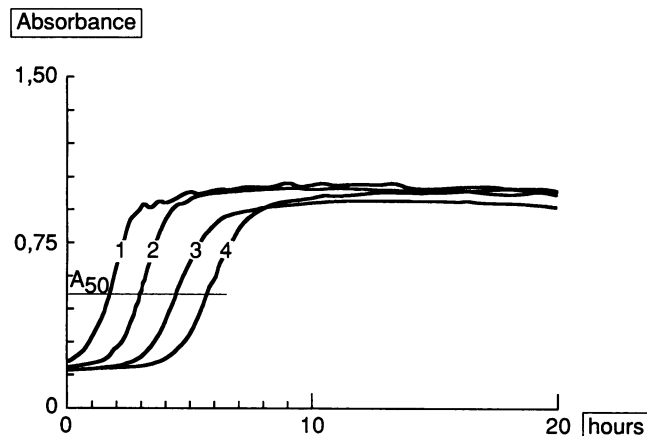


FIG. 1. Control curves of *S. pyogenes* group A isolate U 118 with different inocula. Curves: 1,  $4 \times 10^7$  CFU/ml; 2,  $4 \times 10^6$  CFU/ml; 3,  $6 \times 10^5$  CFU/ml; 4,  $4 \times 10^4$  CFU/ml.

growth, as measured by optical density. The lowest detectable level of optical density for streptococci and pneumococci corresponded to approximately  $5 \times 10^5$  CFU/ml.

(iii) **Induction of the PA phase.** After incubation for 6 h, the cultures were diluted to obtain a bacterial density of approximately  $5 \times 10^7$  CFU/ml. Four-milliliter aliquots were then exposed to 2, 10, and 50 $\times$  MIC of benzylpenicillin for 2 h in 10-ml round-bottomed glass tubes. Control cultures were left untreated. The exposed and control cultures were then washed twice by centrifugation at  $1,500 \times g$  for 10 min each time and diluted in fresh broth to obtain a bacterial density of approximately  $10^5$  CFU/ml. The experiments were performed four times with *S. pyogenes* group A M12 NCTC P1800, three times with *S. pneumoniae* ATCC 6306, and once with the clinical isolates of *S. pyogenes*. In addition, comparative experiments were performed simultaneously with viable counts and Bioscreen C (see below).

(iv) **Induction of the PA SME and SME.** To induce the PA SME, 360- $\mu$ l volumes of benzylpenicillin in broth with a concentration of 0.1 to 0.9 $\times$  MIC were placed in 400- $\mu$ l wells. Samples (40  $\mu$ l each) of the cultures in PA phase were then added to the wells, and the wells were incubated in the Bioscreen C incubator for 20 h at 37 $^\circ$ C. For comparison, the SME was induced by exposing untreated controls to 0.1 to 0.9 $\times$  MIC. The absorbance (optical density) was measured continuously every 10 min during the experiment. The results were processed by the BioScreen C and printed as growth curves. Viable counts of the exposed cultures were performed before antibiotic exposure, after the 2 h of induction, and after washing. Viable counts of the controls were made at the start of the experiments, before and after the dilutions at 2 h. Three different dilutions of the samples were seeded onto blood agar plates and counted for determination of the number of CFU. Only plates with 10 to 1,000 colonies were counted, and the mean values of counts from the three dilutions were used.

(v) **Definitions of the PAE, PA SME, and SME.** The PAE was defined as the difference in the time required for the exposed and unexposed cultures to grow to a chosen point ( $A_{50}$ ) on the absorbance curve.  $A_{50}$  was defined as 50% of the maximum absorbance of the control cultures. The PA SME and SME were likewise defined as the difference in the time required for the exposed culture and the control culture to reach  $A_{50}$ .

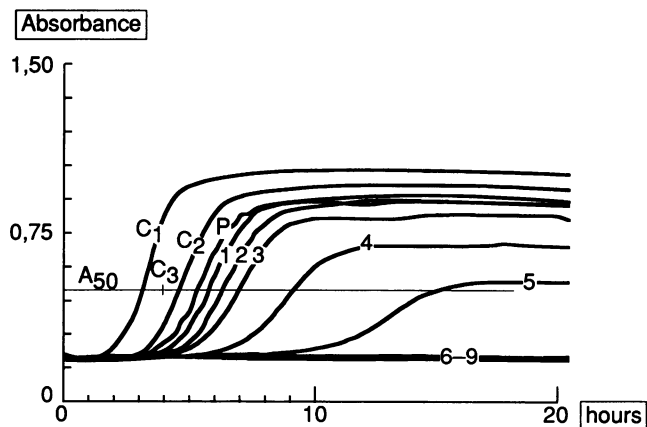


FIG. 2. *S. pyogenes* group A isolate U 118 previously exposed to 10 $\times$  MIC of benzylpenicillin and then reexposed to subinhibitory concentrations (PA SME). Curves: C<sub>1</sub>, control with an inoculum of  $6 \times 10^5$  CFU/ml; C<sub>2</sub>, control with an inoculum of  $4 \times 10^4$  CFU/ml; C<sub>3</sub>, a vertical line indicates where a calculated control, C<sub>3</sub>, having the same inoculum as that of the PA SME cultures ( $10^5$  CFU/ml) reaches  $A_{50}$ ; P, PAE culture, 1 to 9, cultures reexposed to 0.1 to 0.9 $\times$  MIC, respectively.

(vi) **Determination and calculation of the PAE, PA SME, and SME.** At least three different dilutions of the control cultures were made in order to obtain a control with an inoculum as close to the inocula of the cultures exposed to the antibiotic as possible. The growth curves of the controls with different inocula were close to parallel; an example is given in Fig. 1. Therefore, in the experiments where the inocula of the exposed cultures and the controls did not quite match, a control could be constructed with an initial inoculum identical to that of the corresponding exposed strain. The difference in time between the two nearest controls to reach  $A_{50}$  was used to determine when this calculated control should have reached the same absorbance (Fig. 2). The durations of the PAE, PA SME (Fig. 2), and SME (Fig. 3) were then calculated by comparing the growth curve of the exposed culture with that of the control.

**Experiments performed with viable counts.** To compare the Bioscreen results with a conventional technique, additional experiments with *S. pyogenes* group A M12 NCTC P1800

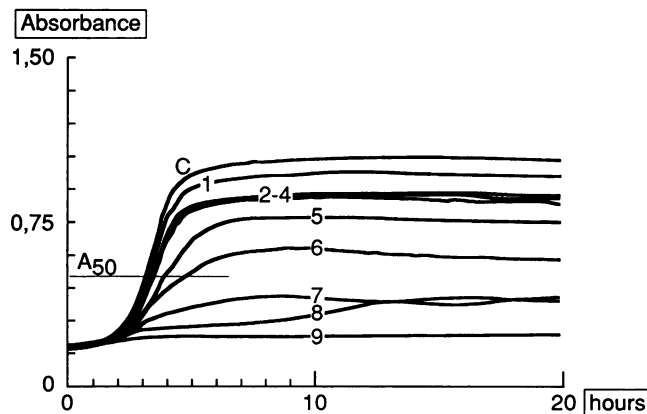


FIG. 3. *S. pyogenes* group A isolate U 118 exposed only to subinhibitory penicillin concentrations (SME). Curves: C, control culture; 1 to 9, cultures exposed to 0.1 to 0.9 $\times$  MIC, respectively.

TABLE 1. PAEs and PA SMEs of benzylpenicillin against *S. pyogenes* and *S. pneumoniae*

Bacterial strain and benzylpenicillin concn used to induce PA	PAE	Mean duration (h) of effect (range)					
		PA SME					
		0.1× MIC	0.2× MIC	0.3× MIC	0.4× MIC	0.5× MIC	0.6× MIC
<i>S. pyogenes</i> group A M12							
2× MIC	2.7 (0.9–3.9)	3.6 (1.5–5.3)	NC <sup>a</sup> (3.6–>20)	>20 (>20)	>20 (>20)	>20 (>20)	>20 (>20)
10× MIC	2.5 (1.9–3.4)	3.3 (2.7–4.3)	5.1 (4.3–6.4)	NC (9.4–>20)	>20 (>20)	>20 (>20)	>20 (>20)
50× MIC	3.2 (2.7–3.6)	3.9 (3.4–4.3)	5.8 (5.0–7.1)	>20 (>20)	>20 (>20)	>20 (>20)	>20 (>20)
<i>S. pneumoniae</i> ATCC 6306							
2× MIC	0.9 (0.8–1.0)	1.4 (1.2–1.6)	1.8 (1.6–2.0)	2.2 (2.0–2.5)	NC (2.7–>20)	NC (3.2–>20)	>20 (>20)
10× MIC	1.5 (0.9–2.7)	1.8 (1.0–2.7)	2.4 (1.9–2.9)	3.5 (3.0–4.1)	NC (3.4–>20)	NC (4.6–>20)	>20 (>20)
50× MIC	1.5 (0.2–2.6)	1.8 (0.9–2.6)	2.4 (1.9–3.2)	3.5 (3.0–4.2)	NC (4.4–>20)	NC (4.8–>20)	>20 (>20)

<sup>a</sup> NC, not calculated because at least one of the experiments yielded a value of >20 h.

and *S. pneumoniae* ATCC 6306 were performed simultaneously with viable counts (two experiments with each strain).

(i) **Determination of MICs.** The MICs were determined with conventional twofold serial dilutions starting with 0.5 mg/liter in Todd-Hewitt broth, inoculated with approximately  $10^5$  CFU of the test strain per ml. The MIC was read after 24 h and defined as the lowest concentration of antibiotic allowing no visible growth.

(ii) **Determination of PAE.** To determine the PAE, a PA phase was induced as described above. The exposed cultures and the controls were then reincubated for 20 h at 37°C in 5% CO<sub>2</sub> in air. Samples were withdrawn every hour for 8 h and at 11 and 24 h. The PAE was defined as  $PAE = T - C$ , where  $T$  is the time required for the viable counts of the antibiotic-exposed cultures to increase 1 log<sub>10</sub> unit above the counts observed immediately after washing and  $C$  is the corresponding time for the unexposed cultures.

(iii) **Determination of PA SMEs and SMEs.** Cultures previously exposed to 10× MIC of benzylpenicillin for 2 h and control cultures were exposed to 0.1, 0.2, 0.3, and 0.4× MIC and incubated for 20 h at 37°C in 5% CO<sub>2</sub> in air. Samples were withdrawn every hour for 8 h and at 11 and 24 h. The effect of sub-MICs on bacteria in the PA phase (PA SME) was defined as follows:  $PA\ SME = T_{pa} - C$ , where  $T_{pa}$  is the time taken for the cultures previously exposed to antibiotic and then exposed to sub-MICs to increase 1 log<sub>10</sub> unit above the counts immediately after washing and  $C$  is the corresponding time for the unexposed cultures. The effect of sub-MICs (SME) on bacteria not previously exposed to antibiotic was defined as  $T_s - C$ , where  $T_s$  is the time for the cultures exposed only to sub-MICs to increase 1 log<sub>10</sub> unit above the counts observed immediately after washing and  $C$  is the corresponding time for the unexposed cultures.

## RESULTS

**Minimum antibiotic concentrations.** The MICs determined with the Bioscreen C incubator were as follows: 0.0156 mg/liter for *S. pyogenes* group A M12 NCTC P1800, 0.0078 mg/liter for the four clinical strains of *S. pyogenes*, and 0.0156 mg/liter for *S. pneumoniae*. The MICs determined with viable counts were always one dilution step higher than those found with the BioScreen C incubator (0.0312, 0.0156, and 0.0312 mg/liter, respectively).

**PAEs and PA SMEs.** The PAEs and PA SMEs for *S. pneumoniae* ATCC 6306 and *S. pyogenes* M12 NCTC P1800 and the clinical isolates are given in Tables 1 and 2. The PA SMEs were always longer than the PAEs. The concentrations used for the induction of the PA phase did not seem to influence the PAEs or PA SMEs (Table 1). This was also true for the clinical isolates (data not shown).

**SMEs.** The SMEs were shorter than the PA SMEs for all bacterial strains studied (Tables 3 and 4). For all experiments in which the sum of the PAE and SME could be calculated (SME <20 h), the sum was shorter than the corresponding PA SME. The SMEs were in all experiments performed with at least three different inocula. At sub-MICs over 0.4× MIC, there was a tendency toward shorter SMEs with higher inocula of the β-hemolytic streptococci. This was, however, not the case with *S. pneumoniae* (Tables 3 and 4).

**Comparison of the Bioscreen C and viable count methods.** There was a good correlation between the two methods when used for determination of PAE and PA SME (Table 5).

## DISCUSSION

The determination of MICs has long been the instrument for expressing the activity of an antibiotic in vitro, although this has often been criticized, since it is a static measurement

TABLE 2. PAEs and PA SMEs induced by 10× MIC of benzylpenicillin against four clinical isolates of *S. pyogenes* group A in a Bioscreen C incubator

Bacterial strain	PAE	Duration (h) of effect							
		PA SME							
		0.1× MIC	0.2× MIC	0.3× MIC	0.4× MIC	0.5× MIC	0.6× MIC	0.7× MIC	0.8× MIC
U 118	1.6	1.9	2.3	3.0	5.3	15.5	>20	>20	>20
U 120	0.9	1.3	1.6	2.4	3.7	7.4	>20	>20	>20
U 121	0.6	1.1	1.4	1.6	1.8	2.6	3.6	5.6	>20
U 123	1.7	2.0	2.5	3.1	5.4	>20	>20	>20	>20



TABLE 3. Effects of subinhibitory concentrations of benzylpenicillin against different inocula of two species

Bacterial strain and inocula (CFU/ml) (no. of expts)	Mean duration (h) of SME (range)					
	0.1× MIC	0.2× MIC	0.3× MIC	0.4× MIC	0.5× MIC	0.6× MIC
<i>S. pyogenes</i> group A M12						
3 × 10 <sup>4</sup> -8 × 10 <sup>4</sup> (4)	0.2 (0-0.3)	0.5 (0-0.9)	1.6 (0.4-2.9)	NC <sup>a</sup> (2.0->20)	>20 (>20)	>20 (>20)
2 × 10 <sup>5</sup> -7 × 10 <sup>5</sup> (4)	0.0 (0.0)	0.1 (0-0.3)	1.1 (0.4-2.0)	NC (1.1->20)	NC (4.5->20)	>20 (>20)
1 × 10 <sup>6</sup> -6 × 10 <sup>6</sup> (3)	0.0 (0.0)	0.1 (0-0.1)	0.7 (0.1-1.7)	NC (0.4->20)	>20 (>20)	>20 (>20)
<i>S. pneumoniae</i> ATCC 6306						
2 × 10 <sup>4</sup> -3 × 10 <sup>4</sup> (3)	0.0 (0.0)	0.2 (0-0.6)	0.4 (0.3-0.4)	0.8 (0.7-1.0)	NC (1.6->20)	>20 (>20)
2 × 10 <sup>5</sup> (2)	0.0 (0.0)	0.2 (0.1-0.2)	0.4 (0.3-0.4)	NC (0.6->20)	NC (1.7->20)	>20 (>20)
9 × 10 <sup>5</sup> -2 × 10 <sup>6</sup> (3)	0.1 (0-0.2)	0.3 (0-0.6)	NC (0.4->20)	NC (0.6->20)	>20 (>20)	>20 (>20)

<sup>a</sup> NC, not calculated because at least one of the experiments yielded a value of >20 h.

and provides limited information with regard to the antibacterial activity of a drug over time. In the clinical situation, when intermittent dosing is used, the concentrations of antibiotics fluctuate and subinhibitory concentrations will often occur between doses. Subinhibitory concentrations of antibiotics are known to have different effects on bacteria. They may alter bacterial metabolism (7), change the cell wall structure (17), and also change the propensity for bacteria to adhere to epithelial cells (21, 22). Furthermore, bacteria exposed to sub-MICs have shown to be more susceptible to leukocyte phagocytosis and killing, the so-called PA leukocyte enhancement (4, 8, 9, 10, 12, 19, 23).

Recently, the effects of low antibiotic concentrations on bacterial growth have attracted a great deal of interest. Sous and Hirsch (20) could demonstrate a prolonged inhibition of staphylococcal growth in an in vitro model for up to 4 h after the concentrations of phenoxymethylpenicillin had fallen below the MIC. Odenholt et al. (13) showed that when streptococci in the PA phase, induced by exposure to 10× MIC of benzylpenicillin for 2 h, were again exposed to 0.1, 0.2, and 0.3× MIC of benzylpenicillin, a long delay occurred before regrowth. This PA SME has also been shown for other antibiotic-bacterium combinations (2, 14-16). The method used for determination of the PA SME and SME in these earlier studies was viable counting. Because this method requires numerous colony counts to be performed, it is both laborious and time-consuming. The purpose of the present study was to investigate whether a new method, using a computerized incubator for bacteria (Bioscreen C), was applicable for this kind of experiment. This method has several advantages: it is more rapid, requires little technician time, allows frequent measurement of the growth of the bacteria, processes the data, and provides a printout. It is also possible to test different bacteria for PAE, PA SME, and SME in the same experiment.

Several investigators have described methods that facilitate the studies of PAE. Gottfredsson et al. (5) used the BACTEC blood culture system that detects bacterial CO<sub>2</sub> by

infrared spectroscopy. In this system the CO<sub>2</sub> absorption measurement was expressed in terms of a growth value. The PAE was calculated by the equation  $PAE = T - C$ .  $T$  was the time required for the cumulative CO<sub>2</sub> production in the exposed organisms to reach a growth value of 30 and  $C$  was the time for the untreated control to reach the same growth value. This method seems to correlate well with the standard method of viable counts. However, the drawback with this method is that the apparatus requires periodic attendance during the whole experiment, while our model does not. Rescott et al. (18) used the Abbot M S-2 research system, a method similar to ours. A 5% decrease in transmittance was used to define the point at which detection growth occurred. To calculate the PAE, they measured the time for 5% decrease in transmittance for the antibiotic-exposed organisms minus the time for the control to decrease the same amount. Gould et al. (6) used a Malthus Microbial Growth Analyser that continuously measures resistance of the growth medium and calculates the change in conductance during growth. The PAE was measured as the difference in the time required for the preexposed bacteria and the control bacteria to reach 10<sup>7</sup> CFU/ml. Baquero et al. (1) investigated the PAE and the bactericidal effects of different antibiotics on different bacteria with continuous impedance monitoring. In our study, we calculated the PAE as the difference in the time required for the exposed culture and the control culture to reach A<sub>50</sub>. The PA SME and SME were calculated similarly. Regardless of which method, the BACTEC system, Abbot M S-2, Malthus Microbial Growth Analyser, or BioScreen C, is used, different dilutions of the control culture must be performed and the dilution closest to the exposed culture must be selected as the control.

In the present study, the MICs used in the Bioscreen C experiments were also measured by examining optical density with the BioScreen C incubator. When *S. pyogenes* M12 and U 118 were challenged with sub-MICs higher than 0.5× MIC, no growth could be demonstrated in the BioScreen C incubator after 20 h, indicating that the true MIC was lower

TABLE 4. Effects of subinhibitory concentrations (SMEs) of benzylpenicillin against four clinical isolates of *S. pyogenes* group A

Isolate (inoculum <sup>a</sup> [CFU/ml])	Duration (h) of SME								
	0.1× MIC	0.2× MIC	0.3× MIC	0.4× MIC	0.5× MIC	0.6× MIC	0.7× MIC	0.8× MIC	0.9× MIC
U 118 (4 × 10 <sup>5</sup> )	0.0	0.1	1.1	>20	>20	>20	>20	>20	>20
U 120 (2 × 10 <sup>6</sup> )	0.0	0	0	0.3	0.2	0.3	1.0	>20	>20
U 121 (2 × 10 <sup>6</sup> )	0.0	0	0	0.1	0.3	0.4	1.1	2.8	>20
U 123 (1 × 10 <sup>4</sup> )	0.3	0.5	1.0	1.8	4.9	>20	>20	>20	>20

<sup>a</sup> Inoculum closest to the inoculum reached after washing or dilution of the corresponding PAE and PA SME experiments (Table 2).

TABLE 5. Comparison of PAEs and PA SMEs of benzylpenicillin against two species by two methods<sup>a</sup>

Bacterial strain, method, and expt no.	Duration (h) of effect				
	PAE	PA SME			
		0.1× MIC	0.2× MIC	0.3× MIC	0.4× MIC
<i>S. pyogenes</i> group A M12					
Bioscreen C					
Expt 1	2.4	2.6	3.3	>20	
Expt 2	2.3	2.3	2.7	>20	
Viable counts					
Expt 1	2.8	3.0	3.8	>11-<24 <sup>b</sup>	
Expt 2	1.7	2.5	2.7	3.8	
<i>S. pneumoniae</i> ATCC 6306					
Bioscreen C					
Expt 1	2.3	2.9	3.4	3.9	
Expt 2	1.8	2.1	2.1	2.5	
Viable counts					
Expt 1	2.8	3.0	3.2	3.2	
Expt 2	1.8	2.5	3.1	>11-<24	

<sup>a</sup> Both species were exposed to 10× MIC of benzylpenicillin to induce PA.

<sup>b</sup> Value unknown but between 11 and 24 h (samples tested at 11 and 24 h).

than determined by twofold serial dilutions. This could explain the high and variable SMEs for *S. pyogenes* M12.

Using the BioScreen C method, we confirmed that streptococci and pneumococci previously exposed to suprainhibitory concentrations of antibiotics were more susceptible to sub-MICs (PA SME) than controls exposed only to sub-MICs (SME). This new method, in contrast to viable counts, permitted easy testing of different bacteria against several antibiotics. However, the definitions of PAE, PA SME, and SME in the BioScreen C experiments had to be different from the definition used in the experiments with viable counts, which normally is defined as the difference in time for the preexposed bacteria and the control bacteria to grow 1 log<sub>10</sub> unit. With the BioScreen C method, the difference in the time required for the bacteria to reach a defined point, A<sub>50</sub> was used instead. Even though the definitions were different, the results were comparable, as seen in Table 5. This is in agreement with earlier studies (13, 14). This new method seems to be convenient for screening different bacteria and different antibiotics for pharmacodynamic parameters, such as, PAE, PA SME, and SME. The results of this study and earlier findings indicate that the subinhibitory concentrations after preexposure to suprainhibitory concentrations may be of importance for the inhibition of bacterial growth of gram-positive bacteria between doses and may be an explanation for the success of intermittent dosing of β-lactam antibiotics.

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