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Postantibiotic Effect of Meropenem on Members of the Family *Enterobacteriaceae* Determined by Five Methods

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The postantibiotic effect (PAE) of meropenem was determined for 11 strains, both clinical isolates and reference strains of members of the family *Enterobacteriaceae*. The study compares PAE results obtained by five methods used to monitor bacterial regrowth, including viable counting, alone and in combination with impedance; bioluminescence, alone and in combination with impedance; and a morphological technique. After exposure of the test organisms to meropenem (0.1× to 100× MIC) for 2 h, concentration-dependent differences in counts by bioluminescence and viable counts were observed, the latter always being lower. The differences varied with the test organism. For example, after exposure of *Providentia stuartii* NCTC 10318 to 0.1× MIC, the counts were 5.5×10^5 and 2.0×10^5 whereas after exposure of *Citrobacter freundii* MR76 to 0.1× MIC of meropenem the counts were 2.3×10^6 and 6.8×10^3 by bioluminescence and viable counting, respectively. The discrepancies were probably due to the relative inability of the viable counting procedure to detect fragile aberrant morphologies and resulted in differences in the calculated PAE values. With methods which do not detect fragile morphologies, the PAE may be underestimated. A general trend was observed for the order of magnitude of the PAEs by the following methods (in order of decreasing magnitude of PAE): (i) morphological technique, (ii) bioluminescence technique alone, (iii) bioluminescence in combination with impedance, (iv) viable counting in combination with impedance, and (v) viable counting alone. It is our opinion that of the methods examined in this study, bioluminescence in combination with impedance best reflects the true values for PAEs, and these results were examined more closely.

Postantibiotic effect (PAE), the delayed regrowth of bacteria after antibiotic exposure, is now a well-established phenomenon that was first described almost 50 years ago (1). The effect appears to be a feature of virtually all antimicrobial agents and has been observed for a wide variety of bacteria and yeasts. It is, however, the specific antimicrobial agent-organism combination which determines the presence and duration of the PAE. Investigators using both old and new β-lactams have consistently observed a PAE with gram-positive cocci (2). In contrast, after exposure of gram-negative bacilli to β-lactams, PAEs, if any, are of short duration or have a negative value. PAE values obtained are, however, very dependent on the methods used to quantify the effect (8, 12). Bearing in mind that gram-negative bacilli generally form fragile spheroplasts when exposed to meropenem, the suitability of performing viable counts on these cell forms has been questioned, and it was found that an underestimation of the number of cells by viable counting led to an underestimation of the PAE (9, 13, 15). This study extends these observations of some members of the family *Enterobacteriaceae* by using an impedance technique (3) in conjunction with a bioluminescence assay of bacterial ATP (6) and compared this technique with viable counting, alone and in combination with the impedance technique; bioluminescence alone; and a morphological technique (8).

MATERIALS AND METHODS

Bacteria, antibiotic, and culture medium. A range of 11 strains of gram-negative bacilli, both clinical isolates and organisms from the National Collection of Type Cultures (NCTC), were used in the study: two strains of *Providentia stuartii* (NCTC 10318 and the clinical isolate MR8), five strains of *Citrobacter freundii* (NCTC 9750 and the clinical isolates MR17, MR18, MR36, and MR76), three strains of *Enterobacter cloacae* (NCTC 10005 and the clinical isolates MR56 and MR75), and one strain of *Escherichia coli* (NCTC 8879). Meropenem was provided by ICI Pharmaceuticals (Cheshire, United Kingdom). Iso-osmotic Malthus Columbia broth (Radiometer, Copenhagen, Denmark) at 546 mosmol/liter was used as the growth medium throughout the study and was achieved by supplementing the broth with sucrose and magnesium sulfate.

Apparatus and parallel procedures. For each strain an inoculum of ca. 10^6 organisms per ml was exposed to five concentrations of meropenem (100×, 10×, 4×, 1×, and 0.1× MIC) for 2 h at 37°C. In parallel, an unexposed control was set up. After the 2-h incubation, each culture was diluted 1:100, and the antibiotic was inactivated by the addition of a broad-spectrum β-lactamase mixture (Genzyme Biochemicals, Kent, United Kingdom). Bioassays were performed from time to time to ensure that the meropenem was inactivated by the β-lactamase, by using the assay organism *E. coli* NIHJ (ICI Pharmaceuticals). We have found that this assay has a lower detection limit of 0.01 μg/ml. Eight tubes containing 10-ml replicates of each of the six cultures were linked to a modified Malthus microbial growth analyzer (Radiometer) (3). The analyzer monitored growth by measuring the resistance of each cell every 6 min and stored the data on a microcomputer (model A440; Acorn Computers Ltd., Cambridge, United Kingdom). The number of cells was determined by a microdilution viable count method (5) at zero hour and 1, 3, and 5 h after antibiotic elimination. Similarly, the bioluminescence assay of ATP (6) was used to determine the number of cells at zero hour and 1, 3, and 5 h after antibiotic elimination. It

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TABLE 1. Comparison of initial killing and PAE results by methods specified for two strains of *P. stuartii* after exposure to meropenem

P. stuartii strain	Meropenem concn[a] (× MIC)	Postexposure counts (no. of organisms/ml)[b]		PAE (h)[c]				
		Bio	VC	Morph	Bio	Imp-Bio	Imp-VC	VC
MR8	100	3.4×10^5	3.9×10^4	2.7	0.7	2.1	0.8	0.5
	10	6.0×10^5	6.8×10^4	2.8	0.4	1.7	0.5	0.2
	4	1.4×10^6	2.0×10^5	2.3	0.1	1.0	0.1	−0.2
	1	2.0×10^6	6.7×10^5	0.1	0.0	0.1	0.1	0.4
	0.1	1.8×10^6	3.8×10^5	0.0	−0.1	−0.1	−0.7	−0.1
	0 (control)	2.1×10^6	7.6×10^5	NA	NA	NA	NA	NA
NCTC 10318[d]	100	$2.4 \times 10^5 \pm 1.7 \times 10^5$	$2.5 \times 10^4 \pm 2.5 \times 10^4$	2.8	0.4 ± 0.4	0.1 ± 0.9	0.3 ± 0.3	0.5 ± 0.3
	10	$4.2 \times 10^5 \pm 3.4 \times 10^5$	$7.3 \times 10^4 \pm 1.3 \times 10^4$	2.70	0.1 ± 0.5	−0.2 ± 0.8	0.1 ± 0.4	0.1 ± 0.2
	4	$4.4 \times 10^5 \pm 3.5 \times 10^5$	$7.7 \times 10^4 \pm 9.4 \times 10^3$	2.7	−0.1 ± 0.5	−0.3 ± 0.9	−0.1 ± 0.3	0.1 ± 0.2
	1	$5.5 \times 10^5 \pm 4.5 \times 10^5$	$7.7 \times 10^4 \pm 1.4 \times 10^4$	0.96	0.2 ± 0.5	−0.4 ± 0.9	−0.2 ± 0.3	−0.2 ± 0.2
	0.1	$5.5 \times 10^5 \pm 4.5 \times 10^5$	$2.0 \times 10^5 \pm 1.3 \times 10^5$	0.00	−0.2 ± 0.5	−0.1 ± 0.3	−0.1 ± 0.1	0.1 ± 0.1
	0 (control)	$9.2 \times 10^5 \pm 2.5 \times 10^5$	$3.6 \times 10^5 \pm 2.8 \times 10^5$	NA	NA	NA	NA	NA

[a] The MICs of meropenem were 0.03 μg/ml for strain MR 8 and 0.12 μg/ml for strain NCTC 10318.
[b] Counts determined by bioluminescence (bio) and viable counts (VC) after meropenem exposure.
[c] PAE determined by a morphological technique (morph) defined as the time required to reach 90% bacilli; by bioluminescence (bio) and applying equation 1 by impedance-bioluminescence (imp-bio) and applying equation 2; by impedance-viable counts (imp-VC) and applying equation 2; and by viable counts (VC) and applying equation 1. NA, not applicable.
[d] Results for strain NCTC 10318 are means ± standard errors of the means.

should be noted that because the bioluminescence assay does not have a low threshold of sensitivity, the zero-hour assay was performed on the cultures immediately after 2 h of antibiotic exposure before the cultures were diluted. The subsequent dilution step was then taken into account (6). This laboratory has established that the bioluminescence assay can detect approximately 10^4 organisms per ml.

For each run, impedance determinations were made for eight replicate tubes, viable counts were carried out in triplicate, and bioluminescence counts were carried out in duplicate. Mean values of these results were used. When replicate runs were carried out on separate days, the data in Tables 1 to 4 have been expressed as the means and standard errors of the means.

PAE determination. For each antibiotic concentration, the PAE was measured by four different methods. Firstly, viable counts were plotted against time, the PAE was calculated as the difference in time, between test and control cultures, for organisms to increase in number by a factor of 10, and equation 1 was applied.

$$\text{PAE} = T - C \quad [1]$$

where T is the time required for the number of organisms in the antibiotic exposed test culture to increase 10-fold after antibiotic elimination, and C is the time required for the number of organisms in the control culture to increase 10-fold (2). Secondly, the counts obtained by bioluminescence were plotted against time, and the equation was again applied. The third and fourth PAE values were computed from the impedance data. Calculation of PAE values by using the Malthus microbial growth analyzer relies on the numbers of bacterial cells at time zero after antibiotic elimination, and these numbers have previously been determined by performing viable counts (3, 4). The PAE value can then be calculated as the difference in time between the test and control cultures to reach 10^7 organisms per ml (after appropriate allowances for differences in their respective inocula have been made). This value is quantified by the following equation:

$$\text{PAE} = [t\,(10^7)\text{e} - t(10^7)_\text{c}] + t_\text{g}'(\ln n_\text{oe}/n_\text{oc}) \quad [2]$$

where $t(10^7)_\text{e}$ is the time taken for the antibiotic-exposed culture to reach 10^7 organisms per ml, $t(10^7)_\text{c}$ is the time taken for the unexposed control to reach 10^7 organisms per ml, t_g' is a function of the generation time (16), n_oe is the number of bacteria present in the test culture after antibiotic exposure and subsequent elimination, and n_oc is the number of bacteria similarly present in the control culture (14).

PAE defined by bacterial morphology. Because of the time-consuming nature of the morphology procedure, it was not carried out in parallel with the other techniques but was carried out on separate days. As described above, an inoculum of ca. 10^6 organisms per ml was exposed to the five concentrations of meropenem for 2 h at 37°C. After incubation, the antibiotic was inactivated by the β-lactamase mixture without dilution of the culture. This was done to ensure that there were sufficient numbers of cells to examine. The cells were observed hourly by interference contrast microscopy on a Neubauer counting chamber. The different morphologies were noted, as were the proportions of the total number of organisms per field. The morphological PAE value was calculated, on the basis of the definition of Lorian et al. (11) and Hanberger et al. (8), as the time taken for the organisms to revert to 90% bacilli and 10% aberrant forms.

RESULTS

All counts determined immediately after the 2-h antibiotic exposure showed discrepancies from the counts determined by bioluminescence and viable counting (Tables 1 to 4). The greater the concentration of meropenem to which the organisms were exposed, the greater the discrepancy between the counts. For the unexposed controls, the counts showed less variation. The degree of variation differed between the organisms.

For all strains tested, the PAE values established by the five different methods showed a consistent trend (Tables 1 to 4). The longest PAEs were obtained by the morphological technique, the highest absolute value detected being 8.4 h for *E. cloacae* MR75 after exposure to 4× MIC. This method, unlike the other methods, gave no negative values, but zero values were recorded on 10 occasions. Immediately after meropenem

TABLE 2. Comparison of initial killing and PAE results by methods specified for five strains of *C. freundii* after exposure to meropenem

C. freundii strain	Meropenem concn[a] (× MIC)	Postexposure counts (no. of organisms/ml)[b]		PAE (h)[c]				
		Bio	VC	Morph	Bio	Imp-Bio	Imp-VC	VC
MR17	100	9.1×10^4	1.2×10^3	3.2	2.5	1.7	−0.1	−0.3
	10	5.0×10^5	1.3×10^4	4.5	1.9	1.6	0.2	−0.1
	4	8.5×10^5	1.5×10^4	4.6	2.0	1.8	0.1	−0.1
	1	8.7×10^5	1.6×10^4	4.6	1.8	1.6	−0.1	−0.3
	0.1	1.0×10^6	3.3×10^4	2.5	1.4	1.5	0.1	0.1
	0 (control)	1.1×10^6	5.3×10^5	NA	NA	NA	NA	NA
MR18	100	4.5×10^6	1.9×10^3	4.8	1.8	3.4	1.3	1.2
	10	4.7×10^6	1.5×10^3	5.2	3.2	3.5	1.2	1.1
	4	5.9×10^6	1.9×10^3	4.0	3.1	3.2	1.0	1.1
	1	5.6×10^6	2.1×10^4	0.0	2.0	2.0	1.0	0.8
	0.1	5.0×10^6	5.3×10^5	0.0	0.1	−0.1	0.5	0.5
	0 (control)	5.6×10^6	1.7×10^5	NA	NA	NA	NA	NA
MR36[d]	100	$4.1 \times 10^5 \pm 5.6 \times 10^4$	$9.9 \times 10^2 \pm 5.9 \times 10^2$	4.1	3.6 ± 0.2	2.9 ± 0.1	0.4 ± 0.1	0.8 ± 0.3
	10	$7.0 \times 10^5 \pm 2.3 \times 10^5$	$1.8 \times 10^3 \pm 8.2 \times 10^2$	4.97	2.5 ± 0.5	2.8 ± 0.2	0.4 ± 0.1	0.4 ± 0.4
	4	$7.4 \times 10^5 \pm 1.9 \times 10^5$	$2.7 \times 10^3 \pm 1.8 \times 10^3$	4.9	2.2 ± 0.7	2.5 ± 0.3	0.20 ± 0.1	0.3 ± 0.4
	1	$7.5 \times 10^5 \pm 1.9 \times 10^5$	$4.7 \times 10^3 \pm 4.0 \times 10^3$	4.83	2.0 ± 0.6	2.3 ± 0.4	0.2 ± 0.1	0.3 ± 0.3
	0.1	$8.3 \times 10^5 \pm 2.5 \times 10^5$	$1.1 \times 10^4 \pm 1.2 \times 10^4$	0.9	0.7 ± 0.4	1.5 ± 0.2	0.1 ± 0.3	−0.2 ± 0.1
	0 (control)	$8.5 \times 10^5 \pm 2.6 \times 10^5$	$6.2 \times 10^5 \pm 1.5 \times 10^5$	NA	NA	NA	NA	NA
MR76[d]	100	$9.0 \times 10^5 \pm 8.8 \times 10^5$	$5.3 \times 10^2 \pm 4.3 \times 10^2$	3.9	>3.8	4.1 ± 0.1	1.5 ± 0.9	2.1 ± 0.9
	10	$5.1 \times 10^5 \pm 3.4 \times 10^5$	$9.6 \times 10^2 \pm 6.4 \times 10^2$	4.0	>3.8	3.5 ± 0.4	1.1 ± 0.9	2.9 ± 0.1
	4	$2.6 \times 10^5 \pm 6.8 \times 10^4$	$1.4 \times 10^3 \pm 9.8 \times 10^2$	3.8	>3.8	3.1 ± 0.8	1.2 ± 1.0	2.6 ± 0.2
	1	$5.9 \times 10^5 \pm 2.8 \times 10^5$	$2.1 \times 10^3 \pm 1.3 \times 10^3$	3.8	>3.8	3.0 ± 0.6	1.0 ± 0.9	2.4 ± 0.3
	0.1	$2.3 \times 10^6 \pm 2.0 \times 10^6$	$6.8 \times 10^3 \pm 6.0 \times 10^3$	0.7	>3.8	2.7 ± 0.8	0.6 ± 1.2	2.2 ± 0.2
	0 (control)	$3.4 \times 10^6 \pm 2.8 \times 10^6$	$7.6 \times 10^5 \pm 7.0 \times 10^5$	NA	NA	NA	NA	NA
NCTC 9750	100	1.4×10^4	1.1×10^3	3.5	−0.7	−0.1	1.3	−1.0
	10	3.4×10^5	3.4×10^3	3.0	1.9	−0.2	0.1	−0.7
	4	6.6×10^5	1.4×10^4	2.9	2.1	−0.4	0.3	−0.3
	1	1.0×10^6	2.0×10^4	2.8	2.3	−0.1	0.6	0.5
	0.1	1.1×10^6	1.6×10^4	0.0	1.7	−0.6	−0.1	−0.1
	0 (control)	9.3×10^7	4.7×10^5	NA	NA	NA	NA	NA

[a] The MICs of meropenem were 0.25, 0.015, 0.06, 0.12, and 0.25 μg/ml for strains MR17, MR18, MR36, MR76, and NCTC 9750, respectively.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.
[d] Results are means ± standard errors of the means.

inactivation and at time intervals thereafter, the numbers of different morphological forms present in the cultures were counted. It is noteworthy that with time the number of spheroplasts of the majority of test organisms declined and that this decrease was not immediately accompanied by a corresponding increase in number of normal bacilli or alternative aberrant forms. Thus, before an increase in the number of bacterial cells was observed, a decline in the number of cells was observed. The next highest values were recorded by the method employing bioluminescence alone. The highest absolute value was recorded 3.6 h for *C. freundii* MR36 after exposure to 100× MIC. By the bioluminescence method alone, only one negative value was obtained, −0.7 h after exposure of *C. freundii* NCTC 9750 to 100× MIC. The combination of impedance and bioluminescence gave mostly positive values, the largest being 5.2 h after exposure of *E. cloacae* MR75 to 100× MIC, and the lowest negative value was −0.6 h after exposure of *C. freundii* NCTC 9750 to 0.1× MIC. Both methods employing viable counting, that is, alone and in combination with impedance, gave the lowest PAE values. Alone, viable counting gave the highest value of 2.9 h after exposure of *C. freundii* MR76 to 10× MIC and the lowest value of −1.0 h after exposure of *C. freundii* NCTC 9750 to 100× MIC. In combination with impedance, viable counting gave the highest value of 1.5 h after exposure of *C. freundii* MR76 to 100× MIC and the lowest value of −0.6 h after exposure of *E. cloacae* MR56 to 10× MIC.

In order to make sense of the vast amount of PAE data in Tables 1 to 4 and to establish the relationship between the different methods employed to determine PAE values, all available PAE values were combined and are presented in Fig. 1. This was achieved by obtaining the mean PAE values for each method, at each concentration for the entire range of test organisms. To establish the relationship between the different methods, the Student *t* test was used. With the test, it was found that there was no statistical difference between the PAE values derived from bioluminescence and bioluminescence-impedance data and between the PAE values derived from viable counting and viable counting-impedance data. There were, however, differences between all other combinations of PAE data. That is, it can be said with 99.9% confidence that the differences in PAE data obtained by bioluminescence and viable counting and by bioluminescence and viable counting-impedance were statistically significant. It can be concluded with 99% confidence that the differences between PAE data obtained by the morphological technique and viable counting,

TABLE 3. Comparison of initial killing and PAE results by methods specified for three strains of *E. cloacae* after exposure to meropenem

E. cloacae strain	Meropenem concn[a] (× MIC)	Postexposure counts (no. of organisms/ml)[b]		PAE (h)[c]				
		OH Bio	OH VC	Morph	Bio	Imp/Bio	Imp/VC	VC
MR56	100	3.7×10^7	3.7×10^3	4.4	1.4	2.8	0.1	0.2
	10	4.7×10^7	3.5×10^3	4.5	2.5	2.2	−0.6	0.1
	4	3.2×10^7	4.7×10^3	4.4	2.1	2.4	−0.1	−0.2
	1	4.3×10^7	1.1×10^3	4.1	1.9	2.3	0.1	−0.1
	0.1	4.2×10^7	3.2×10^4	0.0	1.9	1.8	0.2	0.4
	0 (control)	4.9×10^7	1.1×10^6	NA	NA	NA	NA	NA
MR75	100	9.1×10^5	6.0×10^2	5.6	>4.0	5.2	0.0	1.1
	10	8.9×10^5	6.3×10^2	7.6	>4.0	4.8	−0.3	1.1
	4	1.0×10^6	1.8×10^3	8.4	>4.0	4.7	0.2	0.3
	1	1.6×10^6	4.1×10^3	>8.5	>4.0	4.1	−0.1	0.7
	0.1	2.1×10^6	6.7×10^4	0.0	2.0	1.9	−0.5	−0.6
	0 (control)	2.0×10^6	1.8×10^6	NA	NA	NA	NA	NA
NCTC 10005	100	6.9×10^7	1.6×10^4	4.8	1.6	3.6	0.2	−0.2
	10	6.0×10^7	6.1×10^4	>6.0	3.0	2.7	0.1	−0.7
	4	6.0×10^7	7.0×10^4	>6.0	2.4	2.1	−0.5	−0.7
	1	6.6×10^7	4.8×10^5	>6.0	1.8	1.6	−0.1	−0.3
	0.1	6.0×10^7	5.9×10^6	0.00	0.6	0.2	−0.3	−0.3
	0 (control)	3.7×10^7	9.1×10^6	NA	NA	NA	NA	NA

[a] The MICs of meropenem were 0.06, 0.06, and 0.12 μg/ml for strains MR56, MR75, and NCTC, respectively.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.

by the morphological technique and viable counting-impedance, by bioluminescence-impedance and viable counting, and also by bioluminescence-impedance and viable counting-impedance were statistically different.

Our method of choice is the use of bioluminescence and impedance techniques in combination. By examining this method, relationships between PAE values and the meropenem concentration have emerged. These relationships have been expressed as either quadratic or straight line models (Table 5). We have also provided R^2 values which reflect how the actual data fit the model, with R^2 of 100% reflecting a perfect fit. Only two sets of data do not fit well with the models, namely, the data for the strains of *C. freundii* MR 17 and NCTC 9750.

Table 6 shows the predominant morphologies present immediately after 2 h of exposure to meropenem. Of the 55 organism-meropenem concentration combinations, 25 consisted solely of spheroplasts. Nineteen of the remainder contained spheroplasts with "tails" attached. These were incompletely formed spheroplasts. The alternative morphologies seen were filaments, bacilli, and long bacilli, which could have been filaments.

DISCUSSION

PAE has previously been shown to be method dependent (9, 14, 15). This study set out to show the PAE results obtained for various strains of *Enterobacteriaceae*, both clinical isolates and NCTC strains, after exposure to meropenem, by five different methods, in order to determine which of the five best reflects the PAE and to examine the results obtained by our preferred method.

The preferred method for determining the PAE is the most reproducible method used to monitor the regrowth of the surviving organisms in the PAE phase and that which best correlates with in vivo findings (12). In this study we tried to address the latter of these two points. Until recently, viable counting has been the standard method for monitoring bacte-

TABLE 4. Comparison of initial killing and PAE results by methods specified for one strain of *E. coli* (NCTC 8879) after exposure to meropenem

Meropenem concn[a] (× MIC)	Mean ± SEM counts postexposure (no. of organisms/ml)[b]		PAE (h)[c]				
	Bio	VC	Morph	Bio	Imp-Bio	Imp-VC	VC
100	$4.7 \times 10^6 \pm 4.3 \times 10^5$	$5.5 \times 10^2 \pm 1.8 \times 10^2$	4.7	>3.6	4.1 ± 0.2	0.8 ± 0.1	0.1 ± 0.4
10	$9.1 \times 10^6 \pm 1.6 \times 10^6$	$1.8 \times 10^3 \pm 8.5 \times 10^1$	5.7	>3.6	3.4 ± 0.2	0.3 ± 0.2	0.3 ± 0.5
4	$6.4 \times 10^6 \pm 1.2 \times 10^6$	$3.6 \times 10^3 \pm 2.8 \times 10^2$	5.2	>3.6	2.8 ± 0.2	0.1 ± 0.1	0.2 ± 0.2
1	$8.5 \times 10^6 \pm 2.2 \times 10^6$	$7.4 \times 10^3 \pm 2.0 \times 10^3$	4.7	>3.6	2.3 ± 0.1	−0.2 ± 0.1	−0.1 ± 0.1
0.1	$8.8 \times 10^6 \pm 3.2 \times 10^6$	$5.1 \times 10^4 \pm 6.8510^3$	5.0	2.0 ± 0.1	1.2 ± 0.3	−0.5 ± 0.4	−0.0 ± 0.3
0 (control)	$8.2 \times 10^6 \pm 1.8 \times 10^6$	$4.6 \times 10^6 \pm 2.6 \times 10^6$	NA	NA	NA	NA	NA

[a] The MIC of meropenem for *E. coli* NCTC 8879 was 0.50 μg/ml.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*. Some results are expressed as means ± standard errors of the means.

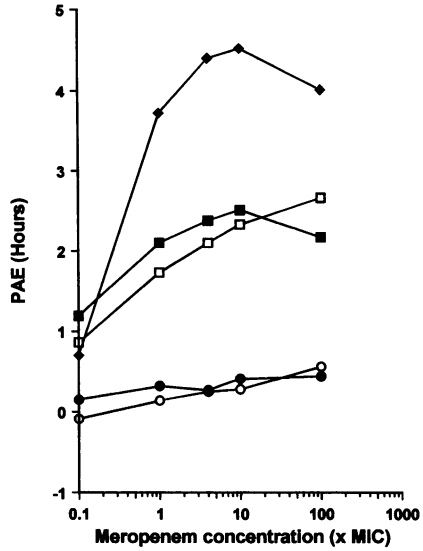

FIG. 1. Mean PAE values determined for the range of test organisms by a morphological technique (◆), bioluminescence (■), bioluminescence-impedance (□), viable counting (●), and viable counting-impedance (○). (Standard errors were less than 10% of the means.)

rial growth, largely for historical reasons. Because of the labor-intensive nature of the method and the inaccuracy of the results when aberrant cell forms are present, however, it is widely accepted that this may no longer be the method of choice. Various alternatives are being offered, and there has been a call for a review of standardization of methods (6, 9, 10, 15, 20). Of the alternatives, we have primarily investigated bioluminescence, alone and in combination with an impedance method, and a morphological technique. Here, we compare them with the standard method of viable counting, alone and in combination with the impedance method.

Tables 1 to 4 present the viable counts and bioluminescence counts determined after 2 h of exposure to five concentrations of meropenem. The results are in accordance with our previous results (13, 14) in showing that the greater the concentration of meropenem, the greater the discrepancy between the counts determined by the two methods. The viable counts were always lower than the bioluminescence counts. The difference in counts determined for the unexposed controls, however, was generally within the accepted 0.5-$\log_{10}$ error inherent with viable counting (16). The discrepancy, however, varies with the bacterial strain and is most likely to be due to the difference in the fragilities between the resultant aberrant morphologies. These results are in accordance with the findings of other investigators, and it would appear that the viable counts were falsely low because of the inability of the procedure to detect fragile cells. Hörnsten and coworkers found that after exposure of a strain of *E. coli* to ampicillin the number of organisms detected was very much lower by viable counting than by bioluminescence and concluded that viable counting does not quantitatively detect spheroplasts (9). In this study, those strains which exhibited the smallest discrepancy over the range of meropenem concentrations of approximately 1 $\log_{10}$ included *P. stuartii* MR8 and NCTC 10318 and *C. freundii* NCTC 9750. It is noteworthy that these strains, unlike the other test strains, did not convert to fully formed spheroplasts after exposure to the range of concentrations of meropenem tested (Table 6). They converted either to filaments or to incompletely formed spheroplasts with tails and would appear to be less fragile than the spheroplasts produced by the other test organisms. It is also noteworthy that in addition to *P. stuartii* NCTC 10318 having the smallest differences between counts by the two methods, this strain is the only strain studied which had no PAE by any method, except the morphological method. By the morphological technique, this strain also had the shortest duration of PAEs of all the strains. Conversely, two organisms in particular showed large discrepancies between counts after exposure to 0.1× MIC, namely, *C. freundii* MR76 and *E. cloacae* MR56. These strains also demonstrated some of the highest PAE values in this study. After exposure to 100× MIC,

TABLE 5. Mathematical models for the relationship between PAE and meropenem concentration for each strain and R^2

Strain	Mathematical model for PAE	R^2 (%)[a]
P. stuartii		
MR 8	0.53 + 0.82 log concn	90.6
NCTC 10318	−0.06 log concn + 0.14 $\log^2$	94.3
C. freundii		
MR 17	1.61 + 0.06 log concn	38.4
MR 18	2.22 + 1.79 log concn	99.2
MR 36	2.27 + 0.62 log concn − 0.15 $\log^2$	99.0
MR 76	2.95 + 0.36 log concn + 0.11 $\log^2$	98.3
NCTC 9750	−0.35 + 0.14 log concn	50.2
E. cloacae		
MR 56	2.15 + 0.29 log concn	81.9
MR 75	3.93 + 1.52 log concn − 0.46 $\log^2$	98.9
NCTC 10005	1.45 + 1.13 log concn	99.0
E. coli NCTC 8879	2.25 + 0.98 log concn	99.0

[a] R^2, the percent variation of PAE explained by the logarithm of the meropenem concentration; 100% reflects a perfect fit of the experimental data with the model.

TABLE 6. Predominant morphologies observed after exposure of all strains to meropenem (0.1× to 100× MIC) for 2 h

Strain	Predominant morphology after exposure to meropenem concn of[a]:				
	100× MIC	10× MIC	4× MIC	1× MIC	0.1× MIC
P. stuartii					
MR8	S (S/T)	S/T (S)	S/T (F)	F	F
NCTC 10318	S	S/T (S)	S/T	S/T	B
C. freundii					
MR17	S	S	S	S (S/T)	S/T
MR18	S	S (S/T)	S (S/T)	S/T	B
MR36	S	S	S	S	F/LB
MR76	S	S	S	S (S/T)	F/LB
NCTC 9750	F/LB (S)	S	S/T	S/T	LB
E. cloacae					
MR56	S	S	S (S/T)	S (S/T)	B
MR75	S	S	S (S/T)	S (S/T)	B
NCTC 10005	S	S	S (S/T)	S/T (S)	B
E. coli NCTC 8879	S	S	S	S	S/T

[a] Parenthetical designations indicate morphologies which were also present but which were not predominate. S, spheroplasts; S/T, incompletely formed spheroplasts with tails; F, filaments; B, bacilli; and F/LB, filaments or long bacilli.

all of the organisms showed large discrepancies between counts, but four strains in particular showed very large discrepancies: *C. freundii* MR 76 and MR 36, *E. coli* NCTC 8879, and *E. cloacae* MR56. At this concentration, these strains gave relatively high PAE values by those methods which did not involve the process of viable counting. Thus, it would appear that a correlation may exist between the aberrant morphology present after antibiotic exposure, the discrepancy between counting methods, and the resultant PAE values.

Spheroplasts may be responsible for the recurrence of symptoms during or shortly after chemotherapy has been stopped (17–19). Thus, spheroplasts may have clinical relevance. As viable counting appears to lyse spheroplasts because of either mechanical forces exerted on them or exposure to an agar surface or to the atmosphere (7), these problems had to be resolved and a more appropriate method of counting had to be found. In iso-osmotic broth, the bioluminescence procedure appears conducive to protecting spheroplasts until such time as the cells are deliberately broken open to release their intracellular ATP for quantitation. It has been suggested, however, that the bioluminescence assay measures the intracellular ATP content not only of live cells but also of intact dead cells (15). This suggests that with the bioluminescence method there may be an underestimation of bactericidal activity and an overestimation of the duration of the PAE. We, however, feel that ATP from this source is minimal and of little significance. It is also possible that large spheroplasts may have a high ATP content, resulting in an overestimation of both the number of cells and the duration of the PAE (8). Further work is necessary to determine how accurately the bioluminescence assay of bacterial ATP quantifies the number of viable bacteria. Impedance monitoring of bacterial growth is also conducive to protecting and quantitating spheroplasts; however, it must be used in conjunction with an alternative counting method.

The ability or inability of counting methods to quantify spheroplasts directly affects the PAE values derived from the relevant data. Hanberger and coworkers found that when large spheroplasts were lysed in water-diluted broth, the PAEs determined by bioluminescence were shortened (8). Utilizing viable counting they also found a more-rapid initial killing and a negative PAE. In this study, therefore, it seems likely that the PAE values derived from viable counts, both alone and in combination with impedance, were falsely low. When bacterial regrowth is monitored by viable counts alone and equation 1 is applied, the counts immediately after antibiotic elimination will be falsely low because of the inability to detect spheroplasts. With time, the surviving cells will become detectable on agar and the increase in the number of cells will appear to be faster than it really is, giving rise to a falsely low PAE value. When the cells in the antibiotic-exposed culture appear to increase in number by a factor of 10 more quickly than those in the control, a negative PAE will result. This was the case with several of our isolates.

The impedance method automatically and accurately monitors bacterial growth (16) but relies on the input of the number of bacteria determined when the cultures go into the system. These numbers are then used directly in the determination of the PAE value by using equation 2. Thus, these numbers must be determined as accurately as possible. In the original impedance work carried out with the Malthus microbial growth analyzer, the inocula were determined by viable counting (3). After exposure of gram-negative bacilli to meropenem, because of the formation of spheroplasts, it was established that this method does not accurately reflect the number of cells in liquid culture, and the more accurate method of bioluminescence is now being used.

Bioluminescence alone was also carried out in this study, and the results are consistent with those found by others who used the method. Comparatively long durations of PAE are found by bioluminescence compared with those by the viable counting and impedance methods. Hanberger and coworkers, however, found that the PAE duration by bioluminescence alone in their study was consistently 1 h longer compared with that obtained by microscopy (8). This was not the case in our present study. In the present study, the PAE values determined by microscopy were similar to those determined by bioluminescence alone and in combination with impedance for some of the test organisms (MR75 and MR76). Most organisms, however, exhibited a longer PAE duration by microscopy than by any of the alternative methods. At the lower concentrations of meropenem, a PAE of 0.0 h was frequently observed by microscopy even though a higher PAE value was determined by bioluminescence alone and in combination with impedance. PAE values of 0.0 h resulted when, after exposure to 0.1× MIC, no aberrant morphologies were found but the cells remained in the form of bacilli. The PAEs determined by microscopy might be expected to correlate well with the results by alternative techniques if the changes in morphology mirror the PAE and if the mechanism of PAE is the same as, or contributes to, the mechanism which causes morphological changes. In fact, this appears to be the case for only two of the test organisms, a finding similar to that of Hanberger and coworkers (8). However, PAE values by bioluminescence in combination with impedance confirm a relationship between morphologically aberrant forms of surviving bacteria and the PAE. We have proposed that of the methods investigated in this study, this method may be the most relevant in assessing PAE values because it may best reflect the in vivo situation (13). Of the methods examined, the combination of bioluminescence and impedance is our method of choice, and we have more fully analyzed the PAE data by this combination of methods.

Mathematical models relating PAE values derived from the bioluminescence-impedance data and meropenem concentration were constructed (Table 5), and the degree of fit between the model and the actual data (Tables 1 to 4) was obtained for each strain. A linear relationship is evident between PAE and meropenem concentration for the strains *P. stuartii* MR8; *C. freundii* MR17, MR18, and NCTC 9750; *E. cloacae* MR56 and NCTC 10005; and *E. coli* NCTC 8879. A quadratic relationship is evident for the strains *P. stuartii* NCTC 10318, *C. freundii* MR36 and MR76, and *E. cloacae* MR75. For 9 of the 11 strains, the actual PAE data fitted well with the models. Two strains, however, did not show a good fit between the PAE data and any model. This was due to what appeared to be aberrant PAE values, which may have become less of a problem had more replicate determinations been carried out.

In conclusion, it appears from the data presented that the PAE values which result from the exposure of members of the family *Enterobacteriaceae* to meropenem are very method dependent. In our opinion, this is largely due to the inability of the standard viable counting method to detect spheroplasts. There was a trend observed. PAE values determined by the morphological technique were higher than those determined by bioluminescence, followed by bioluminescence in combination with impedance, followed by viable counting in combination with impedance, and followed by viable counting. We have chosen to continue using bioluminescence in combination with impedance, as, in our opinion, these techniques are capable of

more accurately quantifying spheroplasts and because automation allows for frequent measurements.

In light of the very method-dependent nature of the PAE values obtained by the different methods, it is necessary to examine carefully the method of choice for particular organism-antibiotic combinations. It is also necessary to standardize the methods in use and to reexamine the appropriateness of maintaining viable counting as the accepted standard method for use in determining PAE. Only when it has been confirmed that we have reliable methods to quantitate PAE can PAE play a role in ensuring that optimum use is being made of both new and established antibiotics.

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