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Flow microcalorimetric assay of antibiotics — II. Neomycin sulphate and its combinations with polymyxin B sulphate and zinc bacitracin on interaction with *Bacillus pumilus* (NCTC 8241)

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Abstract: A flow microcalorimetric assay for Neomycin has been developed which is monitored through interaction of the antibiotic with *Bacillus pumilus* as the test organism. The assay has better reproducibility (relative standard deviation 2.3%) and is more sensitive than conventional microbiological bioassay (0.5–2 $\mu g\ ml^{-1}$). The effects of combinations with zinc bacitracin, with polymyxin B sulphate, and with both zinc bacitracin and polymyxin B sulphate (both in equimolar proportions), and in those proportions present in the commercial preparation Trisep^R (ICI, Macclesfield, UK) have also been investigated. Synergy was observed for the combinations of Neomycin with the other two antibiotics in binary mixtures at the relative proportions found in Trisep^R. The addition of all three antibiotics at the levels used in Trisep^R did not show synergy. However, addition of all three antibiotics at equimolar concentrations did show synergy. It is suggested that microcalorimetry may be useful in *in vitro* experiments for exploring the relative proportions required for maximal effect in antibiotic combinations.

Keywords: *Flow microcalorimetry; polymyxin B sulphate; neomycin sulphate; zinc bacitracin; combined antibiotic therapy; spray-powder preparation; Bacillus pumilus.*

Introduction

This publication is the second in a series [1] describing flow microcalorimetric bioassay of antibiotics (PolB, polymyxin B sulphate; Neo, neomycin sulphate; ZnB zinc bacitracin) utilized in the post-surgery spray preparation Trisep^R (ICI, Pharmaceutical Division, Alderley Park, Macclesfield, Cheshire, UK) and is concerned with the bioassay of Neo. The official pharmacopoeial test organism, *Bacillus pumilus* (NCTC 8241) was used in the development of the assay. An important objective was to investigate the effects of PolB and ZnB on the assay developed.

Neo is an aminoglycoside antibiotic which is believed to exert its action through interaction with the S6 protein of the S30 subunit of the ribosome [2]. Neo appears to have two main effects upon translation [3–9]; (1) inhibition of protein synthesis; and (2)

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induction of errors in translation [10]. Secondary and complex effects accompany the Neo–cell interaction.

Materials and Methods

Organism

Bacillus pumilus (NCTC 8241) was obtained from ICI (Macclesfield, UK) and is the recognized pharmacopoeial strain for Neo assay.

Medium

Bacillus pumilus was maintained on nutrient agar slopes. In growth and calorimetric experiments medium composition was as follows (expressed in g l^{-1} except where stated): glucose, 8 (1.0 in calorimetric incubations); KH_2PO_4, 8.75; K_2HPO_4, 3.75; $(NH_4)_2SO_4$, 2; $MgSO_4 \cdot 7H_2O$, 0.125; MgO, 0.011; $CaCO_3$, 0.002; $FeSO_4 \cdot 7H_2O$, 0.0045; $ZnSO_4 \cdot 7H_2O$, 0.0014; $MnSO_4 \cdot 4H_2O$, 0.0011; $CuSO_4 \cdot 5H_2O$, 0.0002; $CoSO_4 \cdot 7H_2O$, 0.0003; H_3BO_3, 6×10^{-5}. After adjustment to pH 7.9 with HCl the medium was autoclaved (121°, 15 min).

Growth experiments

Growth experiments were performed as described previously [1] at a temperature of 30°.

Antibiotics

Solutions of antibiotics were prepared as described previously [1].

MIC determinations

Minimum inhibitory concentration (MIC) was determined as previously described [1]; at an inoculum level of 1×10^5 colony-forming units (cfu) ml^{-1} the MIC was found to be 0.1 μg ml^{-1} and at an inoculum level of 5.1×10^6 cfu ml^{-1} (the inoculum density used in microcalorimetric incubations) the MIC increased to 0.2 μg ml^{-1}. In both cases the effect was bacteriostatic. Effects of inoculum density on MIC have been noted previously, e.g. for sulphadionidine [11].

Microcalorimetry

The flow microcalorimeter (LKB type 10700-1; 0.45 ml flow-through vessel) its operation and the establishment of the calorimetric incubation were as described previously [12].

Preparation of inocula

Inocula were prepared, frozen and thawed as described previously [1], except that cells, grown at 30° in the medium described above, were harvested when culture dry wt was 1.13 g l^{-1} (corresponding to the late logarithmic phase of growth) and were frozen in quarter-strength Ringer's solution at a rate of 7° min^{-1}. Pre-freeze and post-freeze viable counts were 5.3 ± 0.27 and $3.4 \pm 0.17 \times 10^8$ cfu ml^{-1}, respectively.

Electron microscopy

Freeze-fracture and thin-section electron microscopy were carried out as described previously [15].

Results and Discussion

The glucose concentration used in the microcalorimetric bioassay and growth experiments was selected after examination of a range of concentrations. All concentrations of glucose studied (0.05–0.2%) resulted in a logarithmic increase in power. At the higher organism concentrations obtained with 0.2% glucose, there was a tendency of the bacterium to "clump", thus causing interrupted flow into the microcalorimeter. A concentration of 0.1% glucose gave rise to a logarithmic increase in power up to 16 h 20 min from inoculation. The decline from this peak was shown to be due to glucose exhaustion in the medium. No spores were observed in the period up to the peak. The power–time (p–t) curves were essentially superposable for growth in the presence of 0.1% glucose. The average power at 7 h was 53.5 μW (±2.5%) (Fig. 1).

The two MIC values noted, arise from a dependence upon inoculum density. A pH survey also indicated that Neo was most active at pH 8 (i.e. power was affected most at this pH both in extent and rapidity of interaction). Table 1 and Fig. 2 show the calorimetric consequence of addition of Neo at varying times (corresponding to power developed) through the logarithmic phase. It was convenient, therefore, to assay Neo by addition of the antibiotic at inoculation. Response was recorded during the assay as the difference in power in the presence and absence of the antibiotic 7 h after inoculation/antibiotic addition. Using this definition of response, the assay yielded a linear log dose versus response line over the range 0.25 × MIC (0.5 μg ml^{-1})–1 × MIC (2 μg ml^{-1}). A smaller concentration of Neo gave no response up to 9 h following inoculation. The relative standard deviation (RSD) for one dose was 2.3% (n = 10). Examples of growth curves and of antibiotic-treated incubations are shown in Figs 1 and 2.

The effect of addition of Neo at a power of 39.2 μW (i.e. corresponding to the massive increase in power some 2–4 h later) is presumably a consequence of the detailed molecular events that constitute the mode of interaction of this antibiotic with *B.*

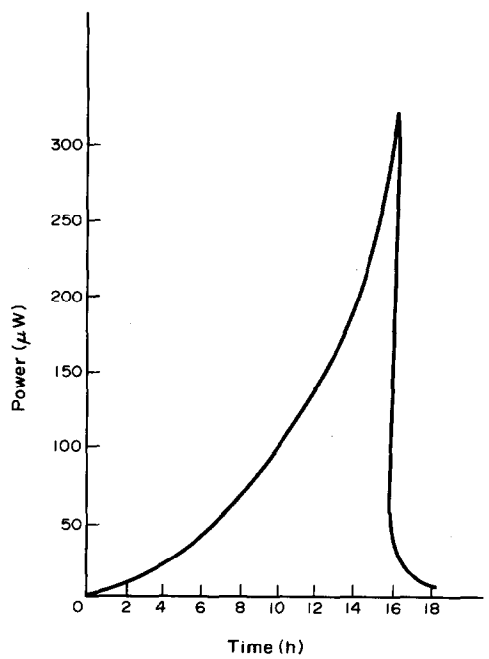

Figure 1
Power–time curve for growth of *B. pumilus* (NCTC 8241) in presence of 0.1% glucose (see Materials and Methods).

Table 1
Effect on power of addition of 0.5 MIC Neo at various power outputs achieved during the logarithmic phase of growth

Power at addition (μW)	Effect
0 (i.e. at inoculation)	Decrease in power from that observed in growth experiment
15.7 23.5 }	Marginal increase in power over normal growth curve
39.2	Massive increase in power 2–4 h following addition

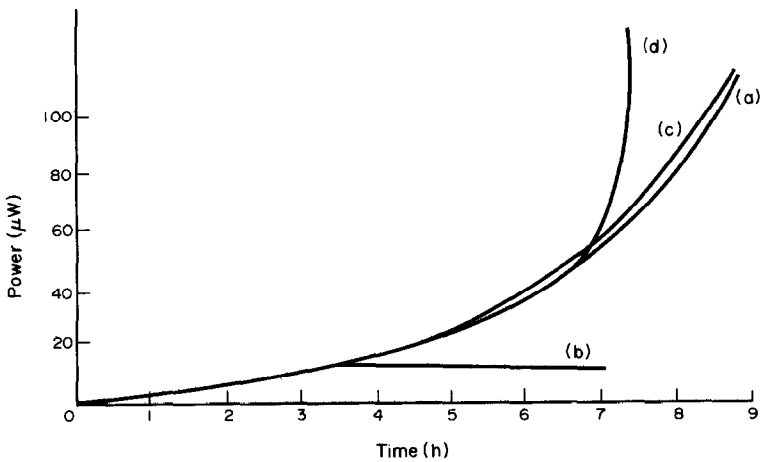

Figure 2
Power–time curves showing the consequences of addition of Neo (0.5 × MIC) at various points during the logarithmic phase of growth (see Table 1). (a) Control, i.e. no added Neo; (b) at inoculation; (c) at point where power is 15.7 or 23.5 μW; (d) at point where power is 39.2 μW.

pumilus. The interpretation of this observation (e.g. misreading or inhibition of protein synthesis) is complex and was not undertaken in this study.

Electron micrographs of fresh and frozen *B. pumilus* and of antibiotic-treated cells were indistinguishable; gross changes in physical form, therefore, do not appear to contribute to the microcalorimetrically observed consequences of the drug–cell interaction.

The MIC values for PolB and ZnB against *B. pumilus* were both 20 g ml^{-1}. At this concentration ZnB had no effect upon the calorimetric incubation (no investigation was made here of any dependence of MIC upon inoculum density with *B. pumilus*). However, PolB when added at a concentration equivalent to the MIC (20 μg ml^{-1}) produced complete inhibition of power at 9 h after inoculation.

Figure 3 shows the p–t curves derived from incubations in the presence of: 0.25 × MIC (0.5 g ml^{-1}) Neo + 0.44 g ml^{-1} (0.022 × MIC) ZnB; and 0.25 × MIC (0.5 μg ml^{-1}) Neo + 0.125 μg ml^{-1} (0.006 × MIC) PolB. These combinations represent the proportions present in the Trisep^{R} preparation. The responses, in both cases, indicate a synergistic interaction equivalent to 0.43 × MIC Neo and to 0.5 MIC Neo, respectively. This type of synergy between aminoglycosides and cell wall/membrane active antibiotics has been

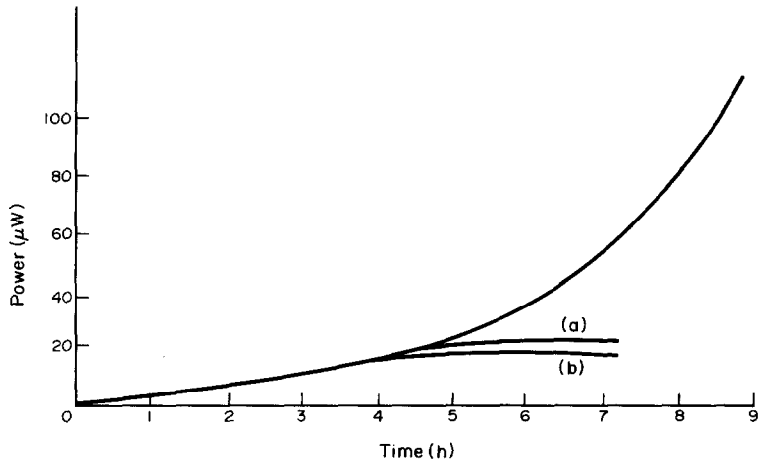

Figure 3
Power–time curves for incubations in the presence of (a) 0.5 μg ml^{-1} Neo + 0.44 μg ml^{-1} (0.022 × MIC) ZnB; and (b) 0.5 μg ml^{-1} + 0.125 μg ml^{-1} (0.006 × MIC) PolB.

well established clinically [13, 14], especially for aminoglycosides and pencillin. These results suggest bacterial membrane disruption by ZnB and PolB, thereby facilitating penetration of Neo into the cytoplasm, thus allowing a more rapid ribosome–antibiotic interaction. A combination of 0.125 μg ml^{-1} (0.0006 × MIC) PolB and 0.44 μg ml^{-1} (0.022 × MIC) ZnB was found to be without effect on the microcalorimetrically monitored growth of *B. pumilus*. It thus appears that these low concentrations of PolB and ZnB, while having some effect on the bacterium, do not have a sufficient effect to alter the p–t growth curves.

No synergistic effect was recorded on addition of all three antibiotics in the proportions present in Trisep^R (0.25 × MIC Neo + 0.006 × MIC PolB + 0.022 × MIC ZnB). However, upon addition of the three antibiotics in equimolar proportions (0.375 × MIC Neo + 0.07 × MIC PolB + 0.08 × MIC ZnB), a response equivalent to 1.75 μg ml^{-1} Neo was recorded, whereas only a 0.75 μg ml^{-1} was actually present. This is a clear indication of synergy and, moreover, in conjunction with the lack of synergism observation for the Trisep^R combinations, indicates that synergy, not surprisingly, may be dependent upon the actual proportions used.

The explanation for these observations is obviously complex. However, it appears that PolB and ZnB when added together at some concentrations may exhibit an antagonistic interaction with *B. pumilus*, whereas at other concentration ratios the effect may be dominated by the interaction effects of, perhaps, only one of these two antibiotics.

These observations suggest the need to investigate a wide range of relative proportions for antibiotic combinations; they also indicate the utility of flow microcalorimetry in revealing this interaction. Such investigations would permit more insight into synergistic activity — at least upon challenge, *in vitro*, with a sensitive organism.

In conclusion, a satisfactory bioassay for Neo upon using *B. pumilus* as the indicator organism has been established. The interaction has been shown to be synergistically affected within some concentration ranges by both PolB and by ZnB. Discrimination between antagonistic and synergistic concentration ranges requires a much more detailed study.

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References

[1] N. Joslin Kjeldsen, A. E. Beezer and R. J. Miles, *J. Pharm. Biomed. Anal.* **7**, 851–857 (1989).
[2] H. E. Carter, J. R. Dyer, P. D. Snow, K. L. Rinehart and M. Hichens, *J. Am. Chem. Soc.* **83**, 3723–3729 (1961).
[3] J. M. Wilhelm, J. J. Jessop and S. E. Pettit, *Biochemistry* **17**, 1149–1153 (1978).
[4] R. R. Goldman and D. B. Northrop, *Biochem. Biophys. Res. Commun.* **69**, 230–236 (1976).
[5] R. Benevista and S. Davies, *Antimicrob. Ag. Chemother.* **14**, 402–409 (1973).
[6] J. Davies and D. S. Smith, *A. Rev. Microbiol.* **32**, 469–475 (1978).
[7] M. S. Bretscher, *Symp. Quant. Biol.* **34**, 651–653 (1969).
[8] B. I. Kanner and D. L. Gutnick, *J. Bacteriol.* **111**, 287–289 (1972).
[9] B. Weisblum and J. Davies, *Bacteriol. Rev.* **32**, 493–528 (1958).
[10] P. Edelman and J. Gallant, *Cell* **10**, 131–137 (1977).
[11] F. Sixl and H. J. Galla, *Biochim. Biophys. Acta* **693**, 466–478 (1982).
[12] B. F. Perry, A. E. Beezer and R. J. Miles, *Microbios* **29**, 81–87 (1980).
[13] J. Klastersky and S. H. Zinner, *Ref. Infect. Dis.* **4**, 294–301 (1982).
[14] G. M. Eliopoulos and R. C. Moellering, *Rev. Infect. Dis.* **4**, 282–293 (1982).
[15] A. E. Beezer and P. B. Sharma, *Microbios* **33**, 161–168 (1982).