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Flow microcalorimetric assay of antibiotics — IV. Polymyxin B sulphate, neomycin sulphate, zinc bacitracin and their combinations with *Escherichia coli* suspended in buffer plus glucose medium

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Abstract: Flow microcalorimetric assays for polymyxin B sulphate and neomycin sulphate have been developed using *Escherichia coli* as the test organism, suspended in a glucose plus buffer medium. These assays have a better reproducibility (relative standard deviations 3.2 and 2.0%, respectively), and require a shorter time (1 h including time required for preparation of the calorimeter) than do conventional microbiological assays, but are not as sensitive. It is suggested that a screening programme might produce a small group of more suitable (i.e. more sensitive) test organisms, which could be used to develop rapid and reproducible flow microcalorimetric assays for a wide range of antibiotics by the procedure described. The effect of combinations of polymyxin B sulphate, neomycin sulphate and zinc bacitracin (the antibiotic components of the commercial preparation Trisep^{R}, ICI, Macclesfield, UK) on the power output of cells suspended in glucose plus buffer medium is also reported. In defined combinations, the effects of neomycin sulphate appeared to be exerted before those of polymyxin B sulphate.

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

Introduction

In previous publications microcalorimetric assays for polymyxin B sulphate (PolB; [1]), neomycin sulphate (Neo; [2]) and zinc bacitracin (ZnB; [3]) have been reported, using *Bordetella bronchiseptica*, *Bacillus pumilis* and *Micrococcus luteus*, respectively. The test organisms chosen were those officially recommended in the British and US Pharmacopoeias for conventional microbiological assays. The assays developed depended on the growth of the test organism in the presence of antibiotic, added either at the time of inoculation (Neo) or during exponential growth (PolB and ZnB). Assay times were in

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the range 4–9 h, and reproducibilities and sensitivities were better than in conventional microbiological assays.

However, if it were possible to develop assays using non-growing, but metabolizing cells (e.g. cells suspended in glucose plus buffer medium), assay times might be considerably reduced. Also, in conventional microbiological assays, different assay organisms are recommended for each antibiotic or related group of antibiotics in the British and US Pharmacopoeias. It would be convenient in laboratories carrying out several assays if the number of assay organisms were reduced. The authors have, therefore, attempted to assay PolB, Neo and ZnB using metabolizing, but non-growing suspensions of *E. coli*. As these antibiotics are components of the commercial preparation Trisep^{R} (ICI Pharmaceutical Division, Alderley Park, Macclesfield, Cheshire, UK) the effects of combinations of these antibiotics on metabolism have also been investigated. It is proposed that such investigations might be useful in indicating synergistic or antagonistic interactions. Flow microcalorimetric studies have previously indicated antagonism between antifungal antibiotics [4].

Materials and Methods

Organisms, medium and preparation of inocula

Inocula of *E. coli* (NCTC 10418) were prepared, frozen and thawed as described previously for *B. bronchiseptica* [1], except that cells were grown on a glucose (0.3% w/v) plus salts medium (pH 7.0, [5]) and harvested when culture dry wt reached 1.0 g l^{-1} (corresponding to the late logarithmic phase of growth). The rate of freezing was 20° min^{-1}. Pre-freeze and post-freeze counts were 3.7 ± 0.6 and $2.5 \pm 0.4 \times 10^{10}$ colony-forming units (cfu) ml^{-1}, respectively.

Antibiotics

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

MIC determinations

Minimum inhibitory concentration values (MIC) were determined by the method described previously [1]. Values obtained for PolB, Neo and ZnB were 6.3, 500 and >1000 μg ml^{-1}, respectively.

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 [6]. The total volume of the incubation medium was 50 ml; the inoculum was 1 ml and incubations were conducted at 37°.

Results

In the absence of antibiotic, the power–time (p–t) curve of *E. coli* inoculated into 0.1 M potassium phosphate buffer (pH 7.0) containing 10 mM glucose showed a rapid increase in power to a plateau value (55 μW), followed after 15 min by a decline to a lower plateau (36 47W) which lasted for several hours (Fig. 1).

Escherichia coli inoculated into glucose plus buffer medium containing PolB gave a peak of power followed by a decline (Fig. 1). The height of the peak and the rate of

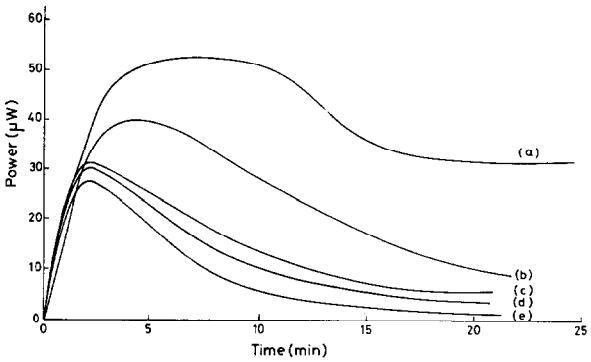

Figure 1
(a) Power–time curves for *E. coli* inoculated into 0.1 M potassium phosphate buffer (pH 7.0) containing 10 mM glucose. Curves (b–e) represent increasing concentrations of PolB (8–50 μg ml^{-1}). (For details, see text.)

decline were dependent upon PolB concentrations. Analysis of p–t curves showed that 8 min after inoculation there was a linear relationship (correlation coefficient 0.9997; gradient 0.34) between power (μW) and log[PolB] for five PolB levels in the range 8–50 μg ml^{-1} (1.3–8 × MIC); relative standard deviations of power RSD; (five determinations) were $<\pm 3.2\%$. There was also a linear relationship (correlation coefficient 0.9987; gradient 1.7) between power (μW) and log[PolB] at 8 min, for lower concentrations of PolB (3–7.5 μg ml^{-1}; 0.5–1.2 × MIC). The sharp change of gradient in the graph of power versus log[PolB] at approximately 8 μg ml^{-1} may indicate a change in the mode of action of PolB.

Escherichia coli inoculated into glucose plus buffer medium containing Neo gave p–t curves which showed a rapid rise in power to a plateau value which was maintained for at least 30 min (Fig. 2). Plateau values were higher than those of control curves (i.e. without antibiotic) and were dependent upon Neo concentration; increasing Neo concentration increased the plateau value. A linear relationship (correlation coefficient 0.996) between the reciprocal of increase in power (compared with control curves) 5 min after inoculation and Neo concentration was found for five Neo levels in the range 20–100 μg ml^{-1} (0.04–0.2 × MIC). The RSDs (n = 5) of the increase in power were $<2.0\%$.

ZnB (300 μg ml^{-1}) did not affect p–t curves of *E. coli* in glucose plus buffer medium. Concentrations higher than 300 μg ml^{-1} were not tested as it was clear that any assay developed using this system would be extremely insensitive.

The p–t curves of *E. coli* inoculated into glucose plus buffer medium containing combinations of PolB (20 μg ml^{-1}), Neo (81 μg ml^{-1}) and ZnB (71 μg ml^{-1}) are shown in Fig. 3 (curves a–e). The ratio of the concentrations used was the same as in the commercial preparation Trisep^{R}. The effect of an equimolar mixture of the three antibiotics (PolB, Neo and ZnB; 19, 10 and 20 μg ml^{-1}, respectively) was also determined (Fig. 3, curve f).

Discussion

Escherichia coli in glucose plus buffer medium gave a power output which was relatively stable with time and which was sufficient to allow the effect of antibiotics on

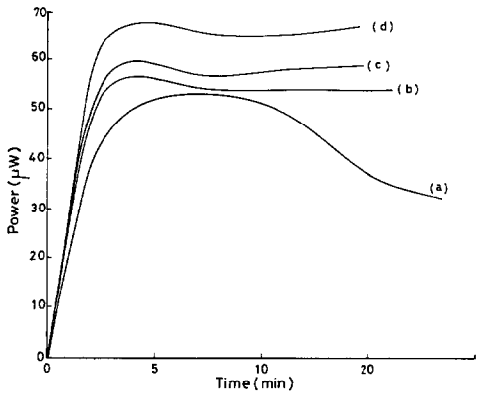

Figure 2
(a–d) Power–time curves for *E. coli* inoculated into 0.1 M potassium phosphate buffer (pH 7.0) containing 10 mM glucose, with increasing concentrations of Neo. (For details, see text.)

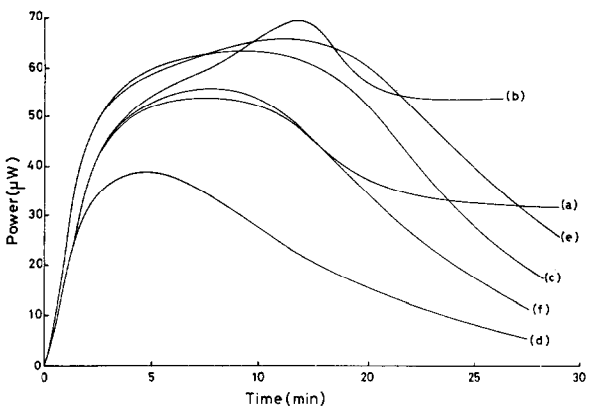

Figure 3
(a–d) Power–time curves for *E. coli* inoculated into 0.1 M potassium phosphate buffer (pH 7.0) containing 10 mM glucose, to which combinations of PolB (20 μg ml^{-1}), Neo (81 μg ml^{-1}) and ZnB (71 μg ml^{-1}) had been added in the same ratio as that for the commercial preparation (Trisep); (f) addition of equimolar ratio of PolB, Neo and ZnB. (For details, see text.)

respiration to be studied. PolB (>0.5 × MIC) decreased power, whereas Neo (0.04–0.2 × MIC) increased power. There have been reports that membrane-active antibiotics [7] and low concentrations of phenol [8] initially stimulate the power output of metabolizing cells. In these cases increased power may have been due to a greater flux of glucose into membrane-damaged cells. However, such an explanation is unlikely for Neo, an inhibitor of mRNA translation. Also, the membrane-active antibiotic, PolB, decreased power output at >0.15 × MIC; lower concentrations were without effect.

The results show that it is possible to rapidly assay PolB and Neo using *E. coli* suspended in glucose plus buffer medium. The assays developed have better reproducibilities (RSD values 2.0 and 3.2% for Neo and PolB, respectively) than conventional microbiological assays; moreover, assay values were obtained with 1 h, including the time required to prepare the calorimeter. The sensitivities of the assays (20 and 8 μg ml^{-1} for Neo and PolB, respectively) were, however, considerably poorer than for microcalorimetric assays of Neo using *B. pumilis* (0.5 μg ml^{-1}, [2]) and PolB using *B. bronchiseptica* (0.4 μg ml^{-1}, [1]), or for conventional microbiological assays using these

organisms. The MIC values of Neo and PolB for *B. pumilis* and *B. bronchiseptica* were both 2 $\mu g\ ml^{-1}$ [9].

The sensitivities of the assays developed are a reflection of the test organisms employed. In this study the authors have used the relatively resistant *E. coli* in the place of *B. pumilis*, *B. bronchiseptica* and *M. luteus* (the other test organisms used in earlier publications [1–3]). This gave low heat outputs when suspended in glucose (or other suitable carbohydrate) plus buffer medium [9]. However, it is envisaged that after a screening procedure, a small group of bacterial strains suitable for the sensitive assay of a wide range of antibiotics by the procedure described here could be found.

The p–t curves showing the effect of combinations of antibiotics (Fig. 3) suggest that ZnB, at a concentration for which it has no effect alone, is antagonistic towards PolB and synergistic to Neo. Curves for PolB plus Neo are complex and show a rapid increase in power to a plateau followed by a decline. The plateau was similar to that expected for Neo alone. However, the gradient of a plot of log(power) versus time, for the decline in power from the plateau, was the same as that for PolB alone. Thus, the effect of Neo on power output was apparently exerted before that of PolB. This preliminary result suggests that PolB and Neo could, perhaps, be assayed together. The two mixtures of all three antibiotics (i.e. Trisep^R combination and equimolar) gave an initial response similar to that expected for Neo alone followed by a decline, presumably due to the effect of PolB.

In conclusion, the authors have examined the possibility of using a single organism (*E. coli*) for rapid flow microcalorimetric antibiotic assays. Assays for PolB and Neo have been developed which require <1 h, and display good reproducibility (RSD 3.2 and 2.0%, respectively), but exhibit low sensitivity. It is suggested, however, that by screening for more suitable organisms, sensitive and rapid assays could be developed for several antibiotics using only one or a small number of bacterial strains and the experimental procedures described.

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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] N. Joslin Kjeldsen, A. E. Beezer and R. J. Miles, *J. Pharm. Biomed. Anal.* **7**, 859–864 (1989).
[3] N. Joslin Kjeldsen, A. E. Beezer and R. J. Miles, *J. Pharm. Biomed. Anal.* **7**, 865–869 (1989).
[4] R. F. Cosgrove, A. E. Beezer and R. J. Miles, *J. Infect. Dis.* **138**, 681–685 (1978).
[5] R. J. Miles and S. J. Pirt, *J. Gen. Microbiol.* **76**, 305–318 (1973).
[6] B. F. Perry, A. E. Beezer and R. J. Miles, *J. Appl. Bacteriol.* **47**, 527–537 (1979).
[7] A. E. Beezer, R. D. Newell and H. J. V. Tyrrell, *Anal. Chem.* **49**, 34–37 (1977).
[8] B. F. Perry, A. E. Beezer and R. J. Miles, *Microbios* **29**, 81–87 (1980).
[9] N. Joslin Kjeldsen, Ph.D. Thesis, University of London (1984).