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# Flow microcalorimetric assay of antibiotics — III. Zinc bacitracin and its combinations with polymyxin B sulphate and neomycin sulphate on interaction with *Micrococcus luteus*

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**Abstract**: A flow microcalorimetric assay for zinc bacitracin has been developed which has better reproducibility (relative standard deviation <2%) and sensitivity (0.02 μg $ml^{-1}$) than conventional microbiological assays, and requires an assay time of between 7.5–9 h. The assay is not suitable for zinc bacitracin determinations in the presence of equimolar concentrations of polymyxin B sulphate or neomycin sulphate, or of these antibiotics in the proportions in which they occur in the commercial preparation Trisep$^R$ (ICI, Macclesfield, UK).

**Keywords**: *Flow microcalorimetry; polymyxin B sulphate; zinc bacitracin; combined antibiotic therapy; spray-powder preparation; Micrococcus luteus.*

## Introduction

This publication is one of a series concerning the application of flow microcalorimetry to the assay of the antibiotic components (PolB, polymyxin B sulphate; Neo, neomycin sulphate; and ZnB, zinc bacitracin) of the post-surgery spray preparation Trisep$^R$ (ICI Pharmaceutical Division, Alderley Park, Macclesfield, Cheshire, UK). Flow microcalorimetric assays have been previously reported for PolB [1] and Neo [2], and these were more sensitive and reproducible than conventional microbiological assays. The present report concerns the development of a microcalorimetric assay for ZnB, and the effect of PolB and Neo on the assay developed.

The test organism used in developing the assay was *Micrococcus luteus* (NCTC 7743), the recognized pharmacopoeial test organism for ZnB assays.

ZnB is a 1:1 complex of $Zn^{2+}$ and bacitracin A, a polypeptide produced by *Bacillus* licheniformis. var. Tracy. It is an inhibitor of cell-wall synthesis and may also affect membrane function [3, 4]. It is active mainly against Gram positive bacteria, but because of its marked nephrotoxicity its use is presently restricted to topical therapy.

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## Materials and Methods

*Organism*

*Micrococcus luteus* (NCTC 7743) was obtained from ICI (Macclesfield, UK); this strain is the recognized pharmacopoeial test organism for ZnB assay.

*Medium*

*Micrococcus luteus* was maintained on nutrient agar slopes. In growth and calorimetric experiments, medium composition (expressed in g $l^{-1}$, except where stated) was: proteose peptone (Oxoid, UK), 10 (4 in calorimetric incubations); sodium citrate, 0.125; biotin, 0.0025; $KH_2PO_4$, 8.75; $K_2HPO_4$, 3.75; $(NH_4)_2SO_4$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.125; $CaCO_3$, 0.002; $FeSO_4 \cdot 7H_2O$, 0.0045; $ZnSO_4 \cdot 7H_2O$, 0.0014; $MnSO_4 \cdot 4H_2O$, 0.0011; $CuSO_4 \cdot 5H_2O$, $2 \times 10^{-4}$; $CoSO_4 \cdot 7H_2O$, $3 \times 10^{-4}$; $H_3BO_3$, $6 \times 10^{-5}$. Peptone and biotin solutions were prepared on the day of use and added to concentrated salt solutions. After adjustment of pH to 7.0 the complete medium was autoclaved (121°, 15 min).

*Growth experiments*

Growth experiments were carried out as described previously [1] except that the temperature was maintained at 30°.

*Antibiotics*

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

*MIC and MBC determinations*

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determinations were carried out as described previously [1]. Both the MIC and MBC were found to be 0.2 μg $ml^{-1}$.

*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 [5].

*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 2.5 g $l^{-1}$ (corresponding to the late logarithmic phase of growth), and were frozen without dimethylsulphoxide at a rate of 7° $min^{-1}$. Pre-freeze and post-freeze counts were $1.75 \pm 0.09$ and $1.70 \pm 0.09 \times 10^9$ colony-forming units (cfu) $ml^{-1}$, respectively.

*Electron microscopy*

Freeze-fracture and thin-section electron microscopy were carried out as described previously [6].

## Results and Discussion

It has been argued previously [1] that for microcalorimetric and other microbiological antibiotic assays, defined media are preferable to undefined media, as they will produce

less variation in bacterial growth. A defined medium containing glucose, salts and various amino acids has been reported for *M. luteus* by Paulton [7]; however, generation times were high (6–17 h) and were reduced only by the addition of yeast extract and protein digests. A defined medium for the strain of *M. luteus* used here was not achieved; although, glucose and pyruvate were tested as possible carbon/energy sources in the presence of salts, biotin and amino acids, including those used by Paulton [7].

Growth was possible in the presence of a number of undefined media components (casein hydrolysate, other protein digests, yeast and liver extracts); the highest yields (>0.5 g dry wt of cells $g^{-1}$ substrate) and specific growth rates (0.2 $h^{-1}$) were obtained in a medium containing salts plus biotin with proteose peptone. This medium also gave a short lag phase (up to 1 h, depending on proteose concentration), and as addition of glucose, pyruvate or yeast extract did not significantly affect the lag phase or specific growth rate, it was chosen for microcalorimetric experiments.

The temperature chosen for microcalorimetric experiments was 30° as this is the optimum growth temperature for *M. luteus* [8]; uptake of ZnB was not markedly affected by temperature [9]. The pH chosen was 7.0; according to the literature this is the optimum for binding of ZnB to phospholipid [10], and was also suitable for the growth of *M. luteus*. Joslin Kjeldsen [9] has shown that uptake of ZnB by *M. luteus* reaches a maximum value within 1 min at pH 7.0, 30°; increasing the pH did not affect the amount of ZnB taken up, but did increase the time required for maximum uptake.

To ensure the reproducibility of inocula in calorimetric experiments, *M. luteus* was stored in liquid nitrogen. Electron micrographs of fresh and liquid nitrogen frozen–thawed cells showed no differences, suggesting that liquid nitrogen freezing caused no gross damage. There were also no differences between electron micrographs of ZnB-treated fresh and frozen–thawed cells; however, even low concentration of ZnB (0.05 μg $ml^{-1}$) induced the formation of rod-like structures in the plasma membrane. These were similar to those previously reported [4] for *Staphylococcus aureus* exposed to high concentrations (100 μg $ml^{-1}$) of ZnB.

Power–time (p–t) curves for *M. luteus* in 0.4% w/v proteose peptone, plus salts and biotin medium, showed an exponential rise in power to an initial peak (92 μW at 7.1 h), followed by a decline and then a further exponential rise (Fig. 1). During the rise in power to the initial peak, there was no change in pH and the cell population increased by approximately four-fold doubling ($8.5 \pm 0.2 \times 10^6$–$1.9 \pm 0.1 \times 10^8$ cfu $ml^{-1}$), corresponding to a specific growth rate of 0.35 $h^{-1}$. This was higher than the growth rates determined in shake-flask experiments and may indicate that proteose peptone contains nutrient(s) in a quantity small enough to be rapidly exhausted by the high populations ($10^8$ cfu $ml^{-1}$) used in these experiments.

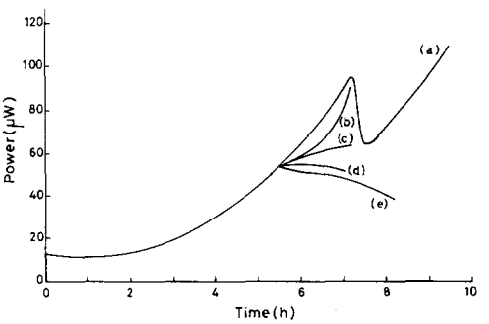


**Figure 1**
Effect of ZnB on p–t curves of *M. luteus* in 0.4% w/v proteose peptone medium. ZnB additions (μg $ml^{-1}$) made when power reached 46 μW were: (a) none; (b) 0.02; (c) 1.5; (d) 2.0; (e) 4.0.

The p–t curves were dependent upon proteose peptone concentration. There was a longer lag phase and the initial peak of power occurred after 10 h when proteose peptone was reduced to 0.2% w/v. As the value of an assay is, in part, dependent upon the assay time required, it was decided to use 0.4% w/v proteose peptone and to make ZnB additions to growing cells at a point corresponding to an increased power output of 46 μW (i.e. one half of that of the initial peak). No attempt was made to characterize the events occurring in p–t curves after the initial peak.

Low concentrations of ZnB (0.1 to 4 × MIC) added to growing cells delayed the initial peak of p–t curves by up to 1 h, whereas higher concentrations (>4 × MIC) decreased power output (Fig. 1). Effects on p–t curves were apparent within a few minutes, presumably reflecting the rapid uptake of ZnB [9] and its activity against cell surface layers (wall and membrane [3, 4]). The ability of sub-MIC levels of ZnB to affect p–t curves is an indication of the sensitivity of the microcalorimetric technique; sub-MIC levels also produced observable changes in cell structure in electron micrographs.

A plot of decrease in power output (μW, as determined from direct measurement of calorimeter voltage output) 20 min after addition of ZnB against log [ZnB] gave a straight line (correlation coefficient 0.99995) for five antibiotic levels in the range 5–20 × MIC (corresponding to 0 to 8 μW). Similarly, for lower ZnB concentrations (five levels in the range 0.1–4 × MIC) a linear relationship (correlation coefficient 0.99987) was found between increase in power output (36 to 6 μW) at 110 min after addition of ZnB and log [ZnB]. Times were chosen to maximize differentiation between p–t curves obtained with differing ZnB concentrations, and to give a useful (i.e. preferably linear) dose–response curve.

Microcalorimetric curves in replicate experiments were almost identical. Relative standard deviations RSD ($n$ = 10) for the increase in power output in control experiments, measured 20 and 110 min after the achievement of a power output of 46 μW, were 0.9 and 1.8% of mean values (6.6 and 45.7 μW), respectively. In the presence of ZnB the RSD values were also <2% for both concentration ranges. The RSD of the assay was thus <2%.

In addition to investigating the effect of adding ZnB to exponentially growing cells, p–t curves were obtained for incubations in which ZnB was added at the time of inoculation. Addition of up to 2 × MIC gave p–t curves in which the initial peak of power was delayed, whereas at 3–5 × MIC there was an almost total inhibition of power output (Fig. 2). There was no correlation between ZnB concentration and calorimetric response. The inability of a concentration corresponding to 2 × MIC to prevent an increase in power output may indicate that that inhibition depends upon cell population.

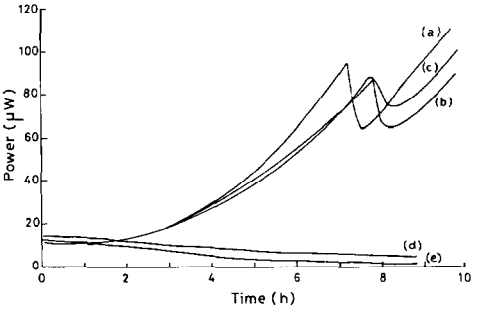


**Figure 2**
Effect of ZnB on p–t curves of *M. luteus* in 0.4% w/v proteose peptone medium. ZnB additions (μg ml$^{-1}$) made at the time of inoculation were: (a) none; (b) 0.2; (c) 0.4; (d) 0.6; (e) 0.8.

The initial population in microcalorimetric experiments ($8.5 \times 10^6$ cfu $ml^{-1}$) was considerably greater than in MIC determinations ($10^5$ cfu $ml^{-1}$).

Attempts were made to assay ZnB in the presence of neomycin and polymyxin, as ZnB occurs with these antibiotics in the commercial preparation Trisep$^R$. It was found that with 0.2 μg $ml^{-1}$ ZnB, together with neomycin and polymyxin in the same proportion as in Trisep$^R$ (i.e. 0.23 and 0.056 μg $ml^{-1}$, respectively), p–t curves after addition of antibiotic (according to the assay procedure given above) showed an increase (4 μW) in the initial peak of power output compared with that for 0.2 μg $ml^{-1}$ ZnB alone. However, the power outputs at the appropriate assay time were identical. This result is, though, fortuitous; ZnB (0.2 μg $ml^{-1}$) with either polymyxin (0.056 μg $ml^{-1}$) or neomycin (0.23 μg $ml^{-1}$) gave an assay response equivalent to only 0.11 μg $ml^{-1}$ ZnB. Similarly, ZnB (0.2 μg $ml^{-1}$) with an equimolar concentration of polymyxin (0.19 μg $ml^{-1}$) and neomycin (0.10 μg $ml^{-1}$) gave an assay value equivalent to 0.10 μg $ml^{-1}$ ZnB.

In conclusion, a microcalorimetric assay for ZnB (0.02 to 4.0 μg $ml^{-1}$) has been developed using *M. luteus* as the test organism. The time required for the assay (7.5 or 9 h depending on ZnB concentration) was shorter and the reproducibility (RSD <2%) and sensitivity (0.02 μg $ml^{-1}$) were better than in conventional microbiological assays. This conclusion is similar to that reached for assay of the other antibiotics reported in this series of papers [1, 2] and is in accord with the findings of Cosgrove *et al.* [11] who compared traditional and microcalorimetric assays for nystatin.

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