A Spectrophotometric Assay of β -Lactamase Action on Penicillins

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A new method for measuring the enzymic hydrolysis of the β -lactam ring in penicillins is described. The change in extinction in the u.v. region is determined. The method is sensitive (50 μ M-benzylpenicillin can be used) and convenient.

For nearly as long as β -lactamases have been known (Abraham & Chain, 1940) the difference between the u.v. absorption of benzylpenicillin and sodium benzylpenicilloate has been on record (Woodward *et al.*, 1949; Mozingo & Folkers, 1949). The difference has hitherto not been used to measure the hydrolysis of penicillins. The present paper describes a spectrophotometric assay of β -lactamases that depends on the difference in u.v. absorption mentioned above. The methods usually adopted for determining the hydrolysis of the β -lactam ring utilize the reaction of the products with iodine, or the reaction of the substrate with hydroxylamine, or continuous titration in a pH-stat (Sabath *et al.*, 1965; Citri, 1971; Ross *et al.*, 1973). Only the last method gives an absolute measure of the extent of reaction. Cephalosporins have a high u.v. absorption which has been used to measure their enzymic hydrolysis (Sabath *et al.*, 1965; Ross *et al.*, 1973), but penicillins lack this absorption. However, penicillins do absorb at lower wavelengths and it is this absorbance that changes on hydrolysis. The same principle has been used to measure hydrolyses catalysed by proteolytic enzymes (Schwert & Takenaka, 1955).

The wavelength used for the spectrophotometric assay was that which gave a maximum in the difference spectrum obtained when unhydrolysed penicillin was scanned against hydrolysed penicillin. The hydrolysed sample was taken as the reference solution for the kinetic runs at fixed wavelength; thus during a run the measured extinction is directly proportional to the concentration of substrate remaining. The proportionality factor is $\Delta\epsilon_{\text{mM}}$ (Table 1); the ratio $\Delta\epsilon/E$, where E is the extinction of the penicillin solution (1 mM), gives the proportional change in u.v. absorption caused by the reaction. This ratio was approx. 0.5 for the penicillins given in Table 1. The method is generally applicable to penicillins, and can be used with crude extracts as well as with purified enzymes.

The spectrophotometric assay was tested with two substrates over a range of concentrations; all the experiments were carried out in 0.05 M-sodium

phosphate, pH 6.8, at 30°C. The concentration of benzylpenicillin was varied from 0.05 mM to 2 mM, and the K_m for the hydrolysis by β -lactamase I from *Bacillus cereus* strain 569/H was 43 μ M; Kuwabara & Abraham (1967) reported 37 μ M. The same enzyme was used for the hydrolysis of 6-aminopenicillanic acid; the concentrations were 0.5–7 mM. This hydrolysis cannot be measured in the pH-stat under neutral conditions (Batchelor *et al.*, 1961a; Sabath *et al.*, 1965), but can readily be determined by the spectrophotometric method. The hydrolysis obeyed Michaelis–Menten kinetics throughout the course of the reaction, and the K_m (1.35 mM) from the course of the reaction (Labia *et al.*, 1973) agreed with the K_m (1.6 $\pm$ 0.2 mM) from the initial rates (v) at substrate concentrations (S); the least-squares fit was to the form: $S/v = S/V_{\text{max}} + K_m/V_{\text{max}}$. Thus this substrate does not inactivate the enzyme, nor does the product inhibit the enzyme. Batchelor *et al.* (1961b) reported a somewhat higher value (3 mM) at a lower pH (5.6). Concentrations of substrate higher than 1 mM were

Table 1. Differences in extinction between penicillins and the corresponding penicilloates

The penicillin (1 mM) in 50 mM-sodium phosphate buffer, pH 6.8, at 30°C was hydrolysed by β -lactamase I from *B. cereus* strain 569/H and the spectra of the unhydrolysed and hydrolysed penicillins compared in cells with a path-length of 1 cm. The second column gives the wavelength (λ) of the maximum in the difference spectrum. The s.d. of ϵ was less than 5% of the mean.

Penicillin	λ (nm)	$\Delta\epsilon$ (l·mmol ⁻¹ ·cm ⁻¹)	E	$\Delta\epsilon/E$
Benzylpenicillin	232	0.94	1.74	0.54
6-Aminopenicillanic acid	223	1.20	1.85	0.65
6-Aminopenicillanic acid	250	0.19	1.29*	0.15
Ampicillin	235	0.67	1.71	0.39
Carbenicillin	235	0.83	1.66	0.50

* This is E_{250} for 5 mM substrate.

analysed at 250nm (Table 1). Initial values of E were kept below 2; a Cecil model 272 grating spectrophotometer (Cecil Instruments Ltd., Cambridge CB4 1TG, U.K.) and a Servoscribe recorder were used.

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Abraham, E. P. & Chain, E. (1940) *Nature (London)* **146**, 837

- Batchelor, F. R., Chain, E. B., Hardy, T. L., Mansford, K. R. L. & Rolinson, G. N. (1961a) *Proc. Roy. Soc. Ser. B* **154**, 498–508
- Batchelor, F. R., Cameron-Wood, J., Chain, E. B. & Rolinson, G. N. (1961b) *Proc. Roy. Soc. Ser. B* **154**, 514–521
- Citri, N. (1971) *Enzymes*, 3rd edn., **4**, 23–46
- Jansson, J. A. T. (1965) *Biochim. Biophys. Acta* **99**, 171–172
- Kuwabara, S. & Abraham, E. P. (1967) *Biochem. J.* **103**, 27c–30c
- Labia, R., Andrillon, J. & Le Goffic, F. (1973) *FEBS Lett.* **33**, 42–44
- Mozingo, R. & Folkers, K. (1949) in *The Chemistry of Penicillin*, (Clarke, H. T., Johnson, J. R. & Robinson, R., eds.), p. 573, Princeton, Princeton University Press
- Ross, G. W., Chanter, K. V., Harris, A. M., Kirby, S. M., Marshall, M. J. & O'Callaghan, C. H. (1973) *Anal. Biochem.* **54**, 9–16
- Sabath, L. D., Jago, M. & Abraham, E. P. (1965) *Biochem. J.* **96**, 739–752
- Schwert, G. W. & Takenaka, Y. (1955) *Biochim. Biophys. Acta* **16**, 570–575
- Woodward, R. B., Neuberger, A. & Trenner, R. N. (1949) in *The Chemistry of Penicillin* (Clarke, H. T., Johnson, J. R. & Robinson, R., eds.), p. 415–439, Princeton, Princeton University Press