Simple Photometric Assay of $\beta$-Lactamase Activity

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A simple photometric assay of $\beta$-lactamase activity was developed. The method is based on a decrease in optical density at 620 nm caused by the formation of a penicilloic acid-iodine complex. The enzymatic reaction is instantaneously stopped by the addition of a concentrated iodine-tungstate solution. Data showing the time and concentration dependence of the reaction are presented. By varying both the time of the assay and the concentration of the enzyme, substrates of widely different $V_{\text{max}}$ values could be assayed. The assay is compared with other methods of determining $\beta$-lactamase activity.

The activity of $\beta$-lactamase has been assayed by a variety of methods (3, 7). Several of these methods are based on the formation of a colorless iodine-penicilloic acid complex (2, 9, 12, 13), and the decrease in iodine is taken as a direct measure of the enzymatic activity. The timed iodometric method (2) has been used extensively in this laboratory; however, this method is unsuitable for measuring iodine-sensitive $\beta$-lactamase activity.

The iodometric titration method of Perret, which overcomes this drawback, is based on back-titration of iodine with thiosulfate. The catalytic reaction is stopped by the addition of a relatively high concentration of iodine at pH 4. (12). Sargent (13) introduced a simplified version of this method in which the optical density of the residual iodine was measured at 490 nm.

However, as was pointed out by Perret (12) and Csányi (9), under certain conditions the catalytic reaction is not completely stopped. Csányi suggested the addition of sodium tungstate and gelatin to the iodine-aceate mixture to stop the reaction more effectively (9).

In this report, the method introduced is based on the above principles. The conditions described, however, result in an immediate and more effective termination of the enzymatic reaction and a more accurate measurement of the residual iodine at 620 nm.

**MATERIALS AND METHODS**

$\beta$-Lactamase. The purified exopenicillinase of *Bacillus cereus* strain 569/H was prepared as previously described (6). All dilutions of the enzyme were made in tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (0.05 m, pH 7.0) unless otherwise stated.

Penicillins. Sodium salt of benzylpenicillin was purchased from Rafa Laboratories. Methicillin (Celbenin batch no. G-437/1) was obtained from Beecham Research Laboratories. Substrates were prepared daily and kept on ice.

**Reagents.** Iodine and potassium iodide (chemically pure grade) were purchased from Agan Chemical Co. Sodium tungstate was a product of British Drug House Ltd.

The iodine-tungstate solution was prepared by diluting the stock iodine solution (0.25 m iodine in 1.25 m KI) 1:3 in 1 m sodium tungstate.

**Assay of $\beta$-Lactamase activity.** Penicillinase activity was determined by the timed iodometric assay (2, 3), a colorimetric assay based on hydroxamate formation (1, 3), and the photometric assay (described below) based on the formation of a colorless iodine-penicilloic acid complex and a corresponding decrease in optical density.

**Photometric assay.** The solutions used were Tris-maleate buffer (0.025 m, pH 7.0), substrate (500 $\mu$moles/ml in buffer), enzyme dissolved in the same buffer as the substrate, and iodine-tungstate solution (0.08 m I$_2$, 0.4 m KI, and 0.67 m sodium tungstate).

Because penicillin is known to adsorb readily to the surface of glass, in other assay procedures gelatin is routinely added to the assay mixture to prevent the adsorption (2). In this assay, the need for gelatin was eliminated by coating the glassware with a 1% silicone solution (Siliclad, Clay Adams, New York, N.Y.). Samples of the enzyme were delivered by means of an automatic micropipette (Eppendorf Mikrofilterpipette) equipped with disposable plastic tips.

The reaction was started by transferring 4 ml of the buffer solution containing 25 $\mu$moles of substrate, prewarmed to 30 C, into the silicone-coated test tube containing the enzyme solution. The reaction was terminated by the addition of 1 ml of the iodine-tungstate solution. The optical density was measured in a Bausch & Lomb spectrophotometer (Spectronic 70) at 620 nm. Control samples, in which the enzyme or substrate, or both, had been eliminated, were included.

One unit of $\beta$-lactamase is defined as the amount of enzyme that hydrolyzes 1 $\mu$ mole of benzylpenicillin/hr at 30 C (4, 12).
RESULTS AND DISCUSSION

One of the essential features of the type of assay described is the ability to inhibit instantaneously the enzymatic reaction. In the case of the β-lactamase system, this is relatively difficult (14). This is not surprising in view of the marked stabilizing effect of typical substrates (5, 8, 10), which protect the enzyme against instantaneous inactivation by protein precipitants such as sodium tungstate or trichloroacetic acid. Similarly, high concentrations of iodine will not alone cause immediate cessation of the catalytic reaction. Additional problems are nonenzymatic hydrolysis of the substrate (e.g., acid hydrolysis by trichloroacetic acid; 14) and "slow nonenzymatic decolorization of iodine" (9, 13). Suggested solutions to these problems were the use of an acid-stable substrate (14) or severe restrictions on the time at which the results can be reliably read (13). Such difficulties and limitations can be avoided if the catalytic reaction is instantaneously arrested without nonenzymatic breakdown of any of the substrates used. In the present procedure, these conditions were met by the use of sodium tungstate and sufficiently high iodine concentrations to stop the reaction. The range of iodine concentrations which is consequently suggested is between 7 and 17 mM. I also found that the optical density of that range of iodine concentrations can be most conveniently measured at 620 nm. This is illustrated in Fig. 1, where the optical density of a range of iodine concentrations determined in a Bausch & Lomb spectrophotometer (Spectronic 70) is shown.

Time dependence of the reaction. Under the conditions described, the kinetics of the penicillinase reaction were found to be linear. This can be seen in Fig. 2, which shows the results when two concentrations of penicillinase were assayed with benzylpenicillin as the substrate.

Concentration dependence. Since the kinetics of the reaction are linear with time, it was possible to use a standard time for assaying various concentrations of the enzyme. Figure 3 shows the results when various concentrations of the enzyme were assayed for 3 min. The optical density of the residual iodine can be directly converted into units of enzyme activity by calculating the number of micromoles of penicilloic acid formed in 1 hr, with the absorption of 4 μmole of iodine per μmole of penicilloic acid assumed (3). This corresponds in the present assay to a decrease of 0.021 optical density unit, as shown in Fig. 1.

Sensitivity of the photometric assay. Although the sensitivity of the assay is limited by the requirement for the addition of fairly concentrated iodine, the range of the assay can be considerably increased by varying the concentration of enzyme used (Fig. 3) or by varying the time of

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**Fig. 1.** Optical density of various iodine concentrations at 620 nm. The optical density of the indicated concentrations of iodine was measured at 620 nm in a Bausch & Lomb spectrophotometer (Spectronic 70). The iodine was dissolved in 5 equivalents of potassium iodide and diluted in 5 ml of Tris-maleate buffer (0.025 M, pH 7.0) containing 0.13 M sodium tungstate.

**Fig. 2.** Kinetics of the penicillinase reaction determined with the photometric assay. Samples of the enzyme containing 143 (▲) and 66 (●) units were assayed with benzylpenicillin (25 μmoles) in 4 ml of Tris-maleate buffer (0.025 M, pH 7.0) at 30 C. The reaction was stopped as described in Materials and Methods at the indicated time intervals, and the optical density was read at 620 nm.
the assay (Fig. 2). By varying both the amount of enzyme and the time of assay, the method was found to be equally reliable for the assay of substrates of widely different $V_{\text{max}}$ values. This is demonstrated in Fig. 4, where the respective rates of hydrolysis of benzylpenicillin and methicillin are compared.

**Comparison of various methods of assay.** In a recent report describing a microiodometric assay of $\beta$-lactamase activity, the authors stated that the uptake of iodine by penicilloic acid is slow relative to the enzymatic reaction (14). This may result in an underestimation of the rate of hydrolysis by 40% (11). To determine whether this slow iodine uptake affected the assay described here, parallel readings were run with the hydroxamate method, which measures the decrease of substrate rather than the accumulation of product. As can be seen in Table 1, under the conditions used, the hydroxamate and photometric assays give similar results. The values obtained with the timed iodometric method were slightly lower, as would be expected because the enzyme preparation was not entirely stable to inactivation by iodine.

**LITERATURE CITED**


**TABLE 1. Comparison of various methods of assay**

<table>
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<tr>
<th>Enzyme (ml)</th>
<th>Timed iodometric assay (units)</th>
<th>Hydroxamate assay (units)</th>
<th>Photometric assay</th>
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**FIG. 3. Constant-time photometric assay.** Samples of the enzyme were assayed with benzylpenicillin (25 $\mu$moles) in 4 ml of Tris-maleate buffer (0.025 M, pH 7.0) at 30 C. The reaction was stopped after 3 min by adding 1 ml of the I$_2$-tungstate reagent. The optical density was read at 620 nm 3 min later. For further details, see Materials and Methods. (○) Optical density at 620 nm. (▲) Units of enzyme activity.

**FIG. 4. Relative rates of hydrolysis of benzylpenicillin and methicillin.** Samples of a penicillinase preparation containing 1,720 units/ml were assayed with 25 $\mu$moles of each of the two substrates, benzylpenicillin and methicillin, for the indicated time intervals at 30 C (see Materials and Methods). The relative rate of hydrolysis of methicillin is 2% of the rate obtained with benzylpenicillin. (■) Enzyme, 0.1 ml; substrate, benzylpenicillin. (○) Enzyme, 0.5 ml; substrate, methicillin.