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Rapid Fixed-Time Assay for Penicillinase

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Many methods of assaying penicillinase activity have been devised (N. Citri and M. R. Pollock, Advan. Enzymol. **28**:237, 1966; N. Citri, Methods Med. Res., **10**:221, 1964; J. M. T. Hamilton-Miller et al., J. Pharm. Pharmacol. **15**:81, 1963; D. A. Wolfe and M. Hamberger, J. Lab. Clin. Med. **59**:469, 1962). These have included colorimetric determinations of acid or alkali production, manometric techniques, direct spectrophotometry, and iodometric assays of penicillin degradation products. Virtually all of these either involve time-consuming rate measurements or have inadequate sensitivity.

In this communication, a simple and rapid fixed-time assay is described that can be adapted for use with most types of colorimetric equipment. The method is evolved from that of C. J. Perret (Nature **174**:1012, 1954) in which iodine strongly buffered to *p*H 4.0 is used to stop the reaction. The product of penicillin hydrolysis, penicilloic acid, reduces iodine, and renders it colorless. The excess iodine is titrated with sodium thiosulfate, and the difference between an assay and blank represents a direct measure of enzyme activity. Although this is probably the best available standard assay, it is extremely time consuming. In the new method, exactly identical conditions are used, but, instead of assaying iodine utilization titrimetrically, the decrease in optical density observed has been used as a measure of enzyme acitivity.

A reaction mixture is prepared containing enzyme in 2.5 ml of phosphate buffer (*p*H 6.5, 0.1 M). After equilibration at 30 C, 10,000 units of penicillin in 0.5 ml of distilled water is added. (For this work, Buffered Potassium Penicillin G, obtained from E. R. Squibb and Sons, New York, N. Y., was preferred, as in solution it is considerably more stable than unbuffered products.) The reaction is stopped after an appropriate incubation period, by adding 5 ml of iodine reagent with rapid mixing. Iodine reagent is prepared by adding 5 ml of stock iodine solution to 95 ml of *p*H 4.0 acetate buffer (80 g of anhydrous sodium acetate adjusted to *p*H 4.0 with acetic acid and made up to 2 liters with distilled water). Stock iodine solution contains 0.32 N iodine and 1.2 M potassium iodide, prepared by dissolving 20.3 g of iodine and 100 g of KI in 500 ml of distilled water.

For a blank, two tubes, one containing buffer and penicillin but no enzyme and another containing only enzyme, are incubated under conditions identical to the assay tubes. After incubation, the contents of these tubes and one 5-ml portion of iodine reagent are mixed. Optical density at 490 mμ is determined after a convenient period of time, and enzyme activity can be expressed in terms of optical-density change per milliliter of enzyme preparation. Superior standardization is obtained by expressing the change in terms of moles of iodine utilized, or in terms of the Perret unit (Nature **174**:1012, 1954). The Perret unit is that quantity of enzyme which hydrolyzes 1 μmole of benzyl penicillin per hr. Perret observed that 1 ml of 0.0166 N sodium thiosulfate was equivalent to 2 μmoles of benzyl penicillin destroyed by penicillinase. By determining the relation of thiosulfate titer to optical density of the iodine reagent, the enzyme activity can be expressed in terms of Perret units.

After the enzyme reaction is terminated by addition of iodine, a slow nonenzymatic decolorization of iodine is observed. However, the rate of decolorization remains virtually constant until over three-quarters of the iodine is decolorized. Furthermore, periods of iodine treatment ranging from 10 to 60 min affect the enzyme assay by less than 5%. The effect of enzyme concentration and length of iodine treatment are illustrated in Fig. 1. It is evident that, providing assay and blank are treated alike, the length of iodine treatment can be varied for convenience within this range without incurring undue error. However, in most experiments iodine treatment was for 30 min. The time course of penicillin decomposition is linear at least until ΔE_{490} reaches 1.0 (Fig. 2); therefore, incubation periods can be varied for convenience.

Typically, for routine assays, a rough and rapid estimate of penicillinase activity is made. Then, for an accurate assay, a dilution is prepared which will give ΔE_{490} of about 0.8 in 30 min. The method can be standardized for a variety of optical equipment, adjusting the wavelength when necessary to keep readings within the reliable range of the instrument. The Gilford 300 automatic sampling spectrophotometer and the Klett-

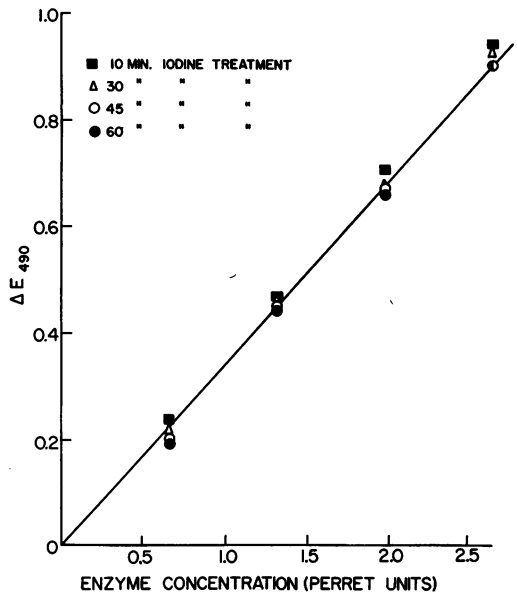

FIG. 1. *Effect of enzyme concentration and iodine treatment on enzyme activity. Reaction mixture is composed of enzyme in 2.5 ml of phosphate buffer (pH 6.5, 0.1 M) and 0.5 ml of penicillin (20,000 units/ml). After 30 min, the reaction was terminated by addition of 5 ml of iodine reagent. ΔE_{490} was determined after iodine treatment shown.*

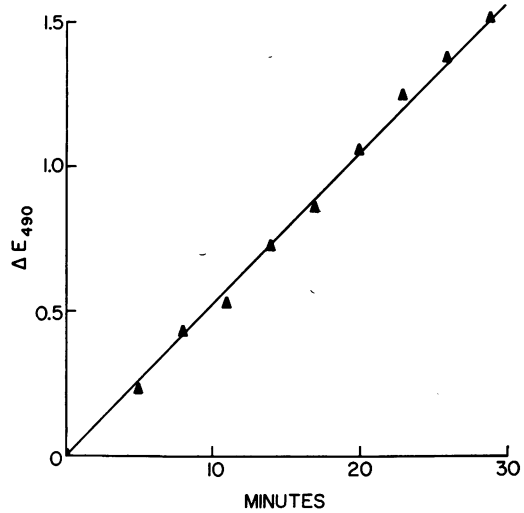

FIG. 2. *Time course of penicillinase action. Reaction mixture as in Fig. 1. Enzyme concentration was 17.5 units. Iodine treatment was for 30 min.*

Summerson colorimeter have been used successfully.

It should be noted that accurate assays can only be made on enzyme preparations containing more than 0.75 Perret unit per reaction mixture with a 30-min incubation period. It is clearly not in the range of sensitivities required for precise determinations of kinetic parameters (R. P. Novick, Biochem. J. **83**:236, 1962). As the reaction conditions are identical to those of Perret and as iodine reduction must necessarily involve decolorization, the relative activities against other penicillins will probably be the same as those obtained by the Perret method (N. Citri and M. R. Pollock, Advan. Enzymol. **28**:237, 1966). The assay is being used as a routine procedure in physiological studies with *Bacillus licheniformis*.

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ERRATUM

Induced Sensitization of Normal Laboratory Animals to *Brucella abortus* Endotoxin

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Volume 95, no. 2, page 286, second column, third line: Change the sentence reading "Attempts to actively sensitize animals to *Brucella* endotoxin by infection other than with live organisms have not generally succeeded." to read "Attempts to actively sensitize animals to *Brucella* endotoxin using other than infection with live organisms have not generally succeeded."