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Microiodometric Determination of β-Lactamase Activity

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The product of β-lactamase activity (a penicilloic acid in the case of a penicillin) is stoichiometrically oxidized by iodine. Hence, the β-lactamase activity can be measured as decolorization of the blue starch-iodine complex. Since the decolorization is a slow process, the rate of decolorization gave an underestimate of the rate of penicillin hydrolysis until a steady state was obtained after 15 to 20 min. If the reaction mixture (3 ml) contained no more than 0.001 unit of enzyme, a correct determination of β-lactamase activity was obtained from the microiodometric method described. The method is sensitive and some applications are mentioned.

There are many methods for assaying β-lactamase activity (1, 3, 4, 7-9), but most of them either employ high substrate concentrations and comparatively high concentrations of enzyme or are laborious to perform. Iodometric methods for β-lactamase assay depend on measuring the amount of iodine needed to oxidize the product of enzyme action (penicilloic acid in the case of penicillin). Routinely, iodine consumption is measured by back-titration with thiosulfate (7, 9), but in the microiodometric method devised by Novick (6) the reaction is followed spectrophotometrically at 620 nm, the absorption maximum of the starch-iodine complex added to the assay at the outset. The Novick microiodometric method has been widely used as it is simple and relatively sensitive. However, Novick reported that the rate of decolorization of starch-iodine underestimated the rate of hydrolysis by 40% (6). We investigated the cause of this discrepancy and found that the rate of decolorization gives a correct estimate of enzyme activity provided that time is allowed for reaching a steady state between the formation of penicilloic acid and its oxidation by iodine. This requires that a pool of penicilloic acid be built up.

MATERIALS AND METHODS

β-Lactamase. β-Lactamase was prepared from *Escherichia coli* that contained the R-factor R_{TEM} as described by Datta and Richmond (2).

Materials. Carbenicillin and ampicillin were kindly provided by Beecham Research Ltd., and benzyl penicillin and cepholosporins by Glaxo Laboratories Ltd., Greenford, England. Hydrolyzed starch (12) was obtained from British Drug Houses Ltd., Poole, England.

Reagents. All reagents were made up in potassium phosphate buffer (0.1 M, *p*H 5.9). Substrates were prepared daily and kept on ice for the period of the experiment. Hydrolyzed starch (0.2%) was dissolved in buffer by boiling gently for 2 to 3 min. Starch-iodine solution was made up by adding 0.15 ml of iodine (0.08 M in 3.2 M potassium iodide) to 100 ml of this starch solution (giving a final iodine concentration of 120 μM).

Analytical procedure. Starch-iodine solution (1 ml) and substrate solution (1 ml) were put in a 1-cm glass cuvette and made up to 2.9 ml with phosphate buffer. The cuvettes were left in the spectrophotometer for 5 min for the temperature to reach 30 C before the reaction was initiated by the addition of enzyme solution (0.1 ml). Absorbance at 620 nm was then measured at various times (see text). The initial absorbance of the normal assay mixture was 1.20.

Units of β-lactamase. One unit of β-lactamase is defined as the amount of enzyme that hydrolyzes benzyl penicillin at the rate of 1 μmole/min. This unit obviously depends upon the temperature at which the assay is performed. All of the experiments reported were performed at 30 C.

RESULTS

Kinetics of the microiodometric assay. In the experiment described in Fig. 1, ampicillin, starch-iodine, and different concentrations of the R_{TEM} β-lactamase were incubated as described in Materials and Methods. The decolorization curves obtained show an acceleration phase before becoming linear after about 15 to 20 min. In general, there was a relationship between the amount of enzyme added and the steepness of the curves, but to be able to use the method for enzyme ki-

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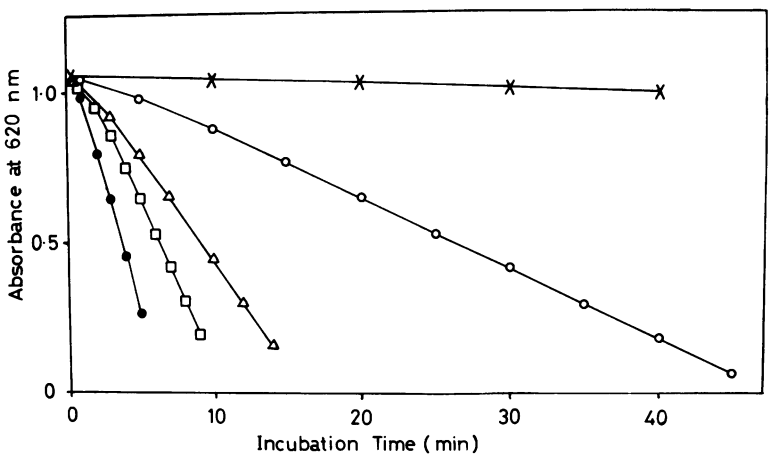

FIG. 1. *Microiodometric assay of* RTEM *β-lactamase with* D*-ampicillin (0.20 mM) as substrate. The amounts of β-lactamase used were 0 (×), 0.5 (○), 2 (△), 4 (□) and 8 (●) × 10^{-3} units.*

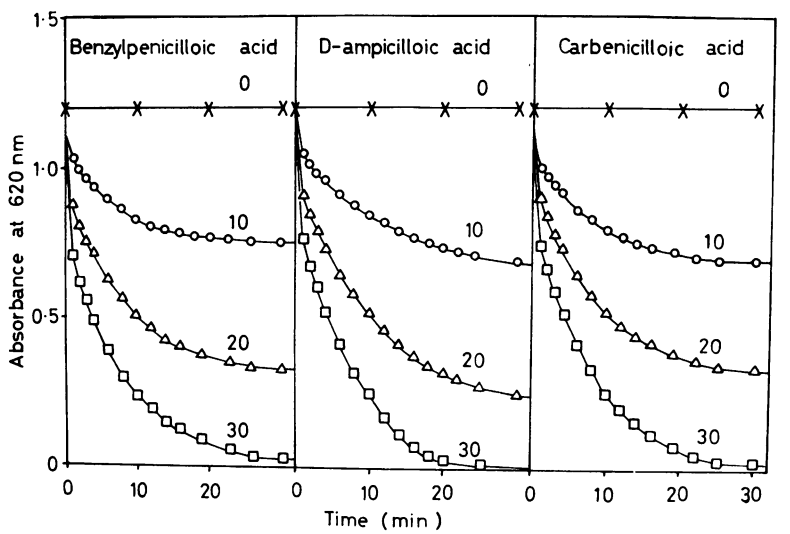

FIG. 2. *Decolorization of starch-iodine by three penicilloic acids. At zero time, 0, 10, 20, or 30 nmoles of penicilloic acids were added to cuvettes containing starch-iodine (1 ml) and phosphate buffer (2 ml). The absorbance at 620 nm was followed. The penicilloic acids were prepared by incubating the penicillins with β-lactamase at 30 C overnight.*

netics it was necessary to find the reason for the nonlinearity in the initial phase and also to see whether the slope of the linear part of the curve could be used as an accurate measure of enzyme rates.

One explanation for the acceleration phase could be that the reaction between penicilloic acid and starch-iodine is slow (Fig. 2). This reaction required 20 min to go to completion. From Fig. 2, it can also be calculated that the oxidation of 1 μmole of penicilloic acid consumed 4 μmoles of iodine and resulted in a decrease in absorbance at 620 nm of 0.040.

To check whether the slow reaction between penicilloic acid and starch-iodine could fully explain the acceleration phase (Fig. 1), a means of instantaneous inhibition of the hydrolysis reaction was required that would not initiate the decolorization reaction. A number of known β-lactamase inhibitors were tested (e.g., methicillin, cloxacillin, and thiol reagents), but these were unsatisfactory either because they did not react fast enough or because they decolorized the starch-iodine. Addition of trichloroacetic acid (0.25%, w/v, final concentration) immediately inhibited the enzyme,

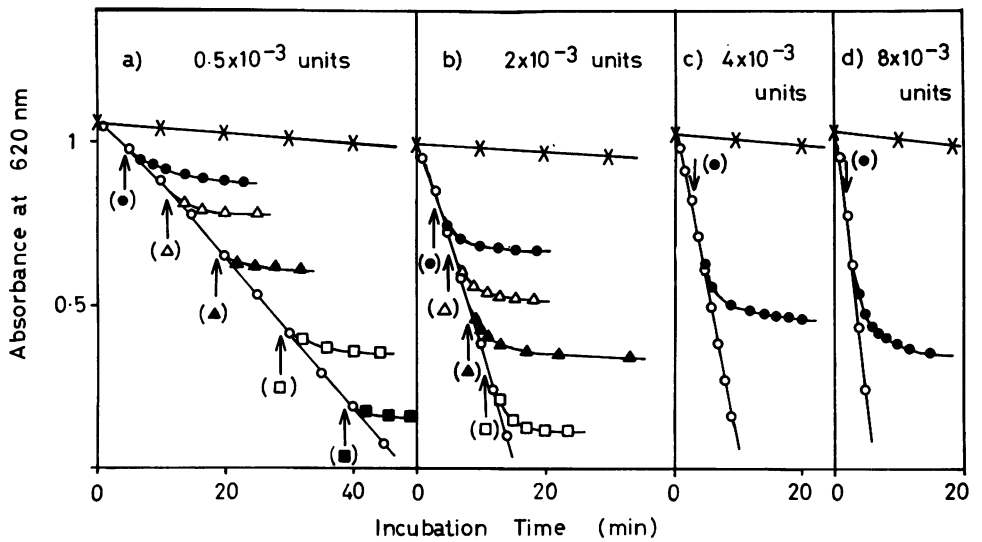

FIG. 3. *Microiodometric assay of β-lactamase with D-ampicillin (0.2 mM) as substrate. Four different concentrations of TEM β-lactamase were assayed, and at intervals trichloroacetic acid was added to stop the reaction as indicated by the arrows. In the control (×), trichloroacetic acid was added immediately before addition of enzyme.*

but obviously this method can be used only with acid-stable penicillins, e.g., ampicillin.

On addition of trichloroacetic acid to an enzyme-ampicillin mixture, decolorization continued for a period before stopping, even though control experiments showed that trichloroacetic acid completely inhibited the enzyme immediately (Fig. 3). The slow decolorization in the controls of Fig. 3 was due to the spontaneous hydrolysis of ampicillin, which was not accelerated by trichloroacetic acid and enzyme (cf. Fig. 1). The decolorization after trichloroacetic acid addition was presumably due to the time required for penicilloic acid to decolorize starch-iodine (Fig. 2).

Thus, addition of trichloroacetic acid allows the total extent of decolorization to be measured and makes it possible to estimate the amount of ampicilloic acid formed during a given period of enzyme action. Figure 3 shows the results obtained on the addition of trichloroacetic acid at various times when four enzyme concentrations were used. The amount of ampicilloic acid formed was found to be a linear function of time (Fig. 4), showing that the nonlinear curves of Fig. 1 cannot be due to any irregularities in the enzyme action. Furthermore, there was a linear correlation between the rate of ampicillin hydrolysis and enzyme concentration (Fig. 5). This means that the decolorization gives a correct estimate of the enzyme reaction and that the reaction can be calibrated with Fig. 2.

Since trichloroacetic acid addition can be used only with acid-stable penicillins, we tested whether the slope of the linear part of the curves in Fig. 1

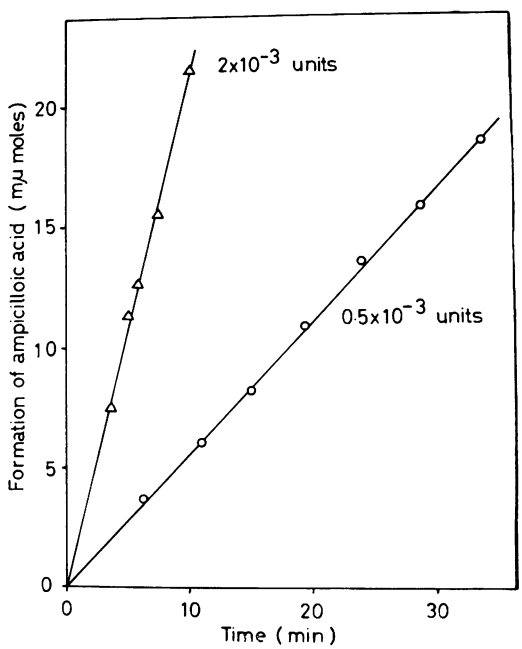

FIG. 4. *Kinetics of formation of ampicilloic acid with the use of two enzyme concentrations. The values for ampicilloic acid have been taken from Fig. 3 by measuring the total decolorization after addition of trichloroacetic acid.*

or 3 could be used to estimate enzyme activity. The slopes were measured at various points on the curves shown in Fig. 3. It is apparent from Table 1 that the slope gives an underestimate of

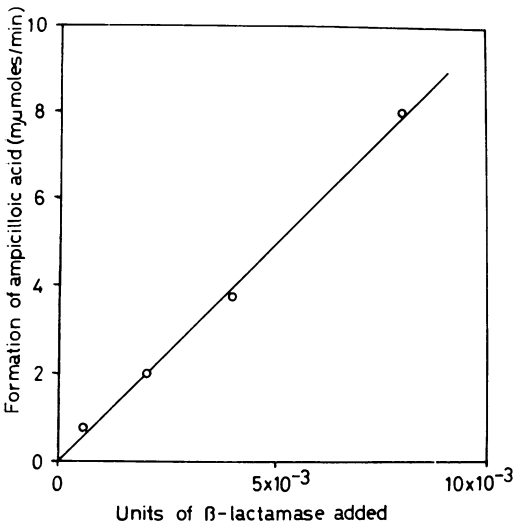

FIG. 5. *Microiodometric assay of β-lactamase. The rate of ampicilloic acid formation (measured after addition of trichloroacetic acid) taken from the experiment described in Fig. 3 has been plotted against the concentration of β-lactamase added.*

TABLE 1. *Estimation of β-lactamase activity from the slope of the decolorization curves of Fig. 1*

β-Lactamase activity (units/3 ml)	Rate of decolorization at different times (% of β-lactamase activity)					
	2.5 min	5 min	7.5 min	10 min	15 min	20 min
0.5×10^{-3}	71	80	89	93	99	100
2×10^{-3}	59	75	81	87	—	—
4×10^{-3}	56	69	73	—	—	—
8×10^{-3}	46	64	—	—	—	—

the real rate of the enzyme reaction in the early part of the assay, but that after 15 to 20 min the slope can be used as a correct measure of hydrolysis rate. Thus, less than 0.001 unit of enzyme should be added to the assay mixture to obtain a reliable result.

The decolorization obtained after addition of trichloroacetic acid is a measure of the concentration of ampicilloic acid. As shown in Fig. 6, the rate of decolorization is directly proportional to the concentration of ampicilloic acid. Hence, the acceleration period in Fig. 1 is used to build up the ampicilloic acid pool until a steady state is obtained between hydrolysis of penicillin and and oxidation of penicilloic acid.

Cephalosporins. Iodine is also consumed during the hydrolysis of cephalosporins, but the decolorization reaction is much slower in such reactions when compared with the hydrolysis of penicillins

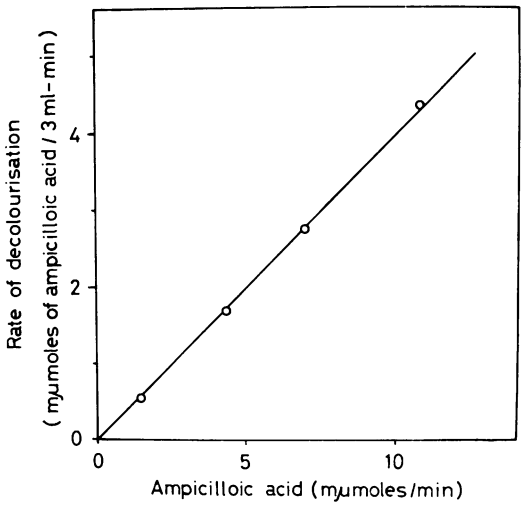

FIG. 6. *Rate of decolorization of starch-iodine as a function of ampicilloic acid concentration. The values were taken from Fig. 3, and the concentration of ampicilloic acid was determined from the extent of decolorization obtained after addition of trichloroacetic acid.*

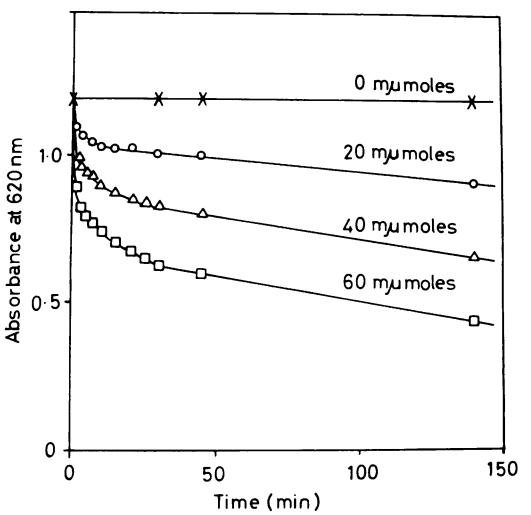

FIG. 7. *Decolorization of starch-iodine by different concentrations of hydrolyzed cephaloridine. At zero time, the acids were added to cuvettes containing starch-iodine (1 ml) and phosphate buffer (2 ml). The absorbance at 620 nm was followed. The cephaloridine was hydrolyzed with β-lactamase.*

(Fig. 7). Also, the iodine consumed per molecule of cephalosporanic acid is much less than is the case with penicilloic acids (9). Figure 8 shows the results obtained by hydrolyzing low concentrations of cephaloridine with enzyme. In this experiment, the molar ratio between the iodine

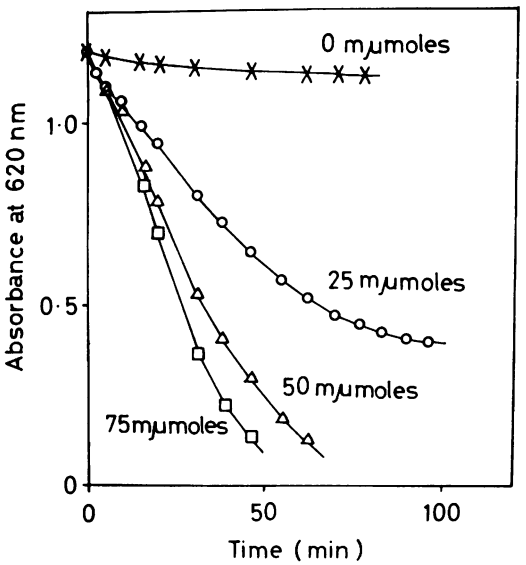

FIG. 8. *Microiodometric assay of β-lactamase with cephaloridine as substrate. The enzyme concentration was 50 times higher than that used in Fig. 3a.*

consumed and the acid added was found to be 3. β-Lactamases usually have a high Michaelis constant for cephalosporins (2, 10, 11), so that the substrate concentrations required for enzyme saturation are at least 1 mM. At this concentration, the spontaneous hydrolysis of cephalosporins is so rapid that accurate estimations of cephalosporinase activity are difficult to achieve by this method.

DISCUSSION

In the microiodometric assay of β-lactamase described in this paper, the rate of hydrolysis of the substrate (reaction A in Fig. 9) is measured by use of the reaction between the product (penicilloic acid in the case of penicillin) and iodine, which is taken from a starch-iodine complex (reaction B in Fig. 9). By utilizing an acid-stable substrate (ampicillin), it has been shown that the hydrolysis reaction proceeds linearly with time (Fig. 4). However, the decolorization of starch-iodine showed an acceleration phase before it became linear after 15 to 20 min (Fig. 1). The slope of the linear part of the decolorization curves gives a correct value of the rate of hydrolysis. The acceleration phase can be fully explained by the fact that reaction B is slow (Fig. 2). Thus, it is necessary to allow time for the steady state between reactions A ad B in Fig. 9 to be reached. The 40% discrepancy between the microiodometric method and other assay methods reported by Novick is almost certainly due to the fact that he allowed only 5 min to reach the steady state (cf. Table 1).

The amount of iodine used in the assay corresponds to 30 nmoles of penicillin. Thus, a correct determination of β-lactamase is obtained for activities up to 0.001 unit per assay mixture. At higher activities, the starch-iodine is completely decolorized before the steady state is achieved.

Cephalosporins. β-Lactamases are also capable of hydrolyzing the β-lactam bond of cephalosporins but, whereas the reaction product with penicillins is stable, cephalosporins form unstable products (5). From Fig. 6, it can be seen that in the case of cephalosporins the decolorization reaction is much slower than with penicillins. Furthermore, the iodine equivalent of cephaloridine was different when hydrolyzed before the addition of starch-iodine (Fig. 7), or hydrolyzed in the presence of starch-iodine (Fig. 8). It is therefore difficult to calibrate the microiodometric method for use with cephalosporins.

Applications of the method. The sensitivity of the method allows the determination of low activities of β-lactamases in crude enzyme preparations and even in cell cultures. We have experienced no difficulty in detecting enzyme activities down to 0.0004 unit per mg of dry weight in gram-negative bacteria. The method is also very useful in kinetic studies because the Michaelis constants of β-lactamases are often very low. The method can also be utilized by leaving reaction mixtures for given periods of time after which decolorization is measured, e.g., when assaying column fractions for enzyme activity or cultures of organisms. Molecular weight determinations of Sephadex G-75 can be achieved by use of 0.2 unit of enzyme. The method has also been automated (B. E. Lindström, *personal communication*).

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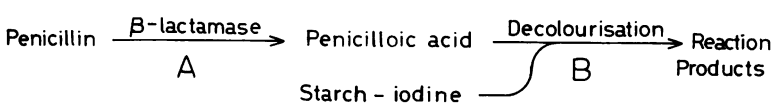

FIG. 9. *Schematic representation of the reactions involved in microiodometric assay of β-lactamase activity.*

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