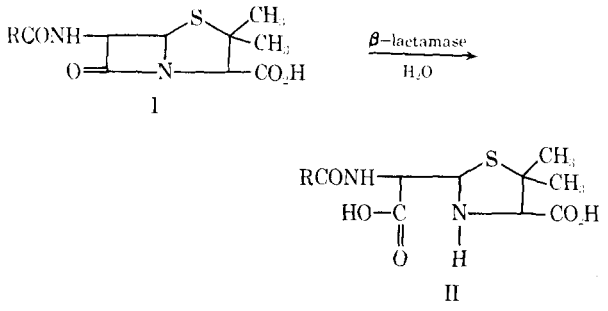
# Measurement of β-Lactamase Activity and Rate of Inactivation of Penicillins by a pH-Stat Alkalimetric Titration Method

JOSEPH P. HOU* and JOHN W. POOLE▲

**Abstract** □ The pH-stat alkalimetric titration method was shown to be applicable and superior to other chemical methods in the investigation of β-lactamase activity as well as in kinetic studies. For both staphylococcal and bacillary β-lactamases, the pH optimums depended not only on bacterial strain and temperature but also on the substrate. Of all the compounds studied, 6-[2-amino-(3-trienyl)acetamido]penicillanic acid, 6-[2-(1-aminocyclohexyl)acetamido]penicillanic acid, 6-(2-amino-2-methylpropionamido)penicillanic acid, and ampicillin were the least stable toward *Staphylococcus aureus* Tex-2 β-lactamase. Penicillin G, phenoxymethyl penicillin, phenethicillin, 6-(1-amino-3-cyclopentene-1-carboxamido)-penicillanic acid, 6-(2-amino-2-indancarboxamido)penicillanic acid, and carbenicillin were about twice as stable as ampicillin, and 6-(1-aminocyclopentanecarboxamido)penicillanic acid, 6-[2-(1,4-cyclohexadien-1-yl)acetamido]penicillanic acid, and cyclacillin were about four times as stable. *N*-Sulfonylation of cyclacillin gave a product, 6-[1-(2-mesitylenesulfonamido)cyclohexanecarboxamido]-penicillanic acid, that was about 10 times as stable as the parent compound. The nature of the penicillin side chain, particularly the stereospecificity, plays a significant role in the stability of this molecule toward the β-lactamases.

**Keyphrases** □ Penicillins—β-lactamase activity, rate of inactivation, pH-stat alkalimetric titration □ Ampicillin—β-lactamase activity, rate of inactivation, pH-stat alkalimetric titration, compared to other penicillins □ Cyclacillin—β-lactamase activity, rate of inactivation, pH-stat alkalimetric titration, compared to other penicillins □ β-Lactamase activity, penicillins, ampicillin, cyclacillin—determination, pH-stat alkalimetric titration □ pH-stat alkalimetric titration—measurement of β-lactamase activity, rate of inactivation, penicillins

Penicillin β-lactamases (penicillinases) are bacterial enzymes capable of hydrolyzing the β-lactam ring of susceptible penicillins (I) to the corresponding biologically inactive penicilloic acids (II) (Scheme I) (1, 2). β-Lactamases are distinct from penicillin amidases, which selectively split the side-chain peptide linkage. Similarly, another type of bacterial enzyme, the cephalosporin β-lactamases (or cephalosporinases), hydrolyzes the cephalosporins to cephalosporoic acid. The β-lactamases are produced by a wide variety of microorganisms, both Gram-positive and Gram-negative, either constitutively or as a result of exposure of the microorganism to an inducing agent such as one of the antibiotics. The growing resistance of many bacteria to the β-lactam antibiotics (*i.e.*, to the penicillins and cephalosporins) may be due to their increasing ability to elaborate the β-lactamases.




*Scheme I*

The distribution, activity, and biochemical properties of these enzymes were recently reviewed by Pollock (3–5), Jack and Richmond (6), Rauenbusch (7), Citri and Pollock (8), and Smith *et al.* (9).

Many chemical methods (10–23) have been used to investigate β-lactamase activity and to determine the susceptibility of the penicillins and cephalosporins to the β-lactamases. Past methods relied heavily on the hydroxylamine (11–13), iodometric (14, 15), microiodometric (16), and manometric (17, 18) assays; less frequently used methods employed optical rotation (19, 20), alkalimetric titration (21–23), and oscillographic polarography (10).

Unfortunately, the manometric (18, 24–32) and hydroxylamine (12, 33–35) assays are tedious and time consuming, while the iodometric (14, 15) and revised iodometric (16) methods are quite dependent on pH and temperature. Moreover, the buffer systems required for these assays unavoidably introduce many extraneous interactions between enzyme, reagent, and substrate. Brief discussions of these methods were given by Citri (36) and Hamilton-Miller *et al.* (37).

Murtaugh and Levy (21), noting that the β-lactamase inactivation of penicillin generates penicilloic acid, a product which theoretically should be quantitatively neutralizable by a standard base, devised a method for determining the purity of a penicillin by alkalimetric titration at a constant pH. Patterson and Emery (22) and Wise and Twigg (23) reported this type of behavior for the *Bacillus subtilis* β-lactamase, and Banfield (38) studied the *B. subtilis* β-lactamase kinetics at several pH values. In the days when the pH-stat alkalimetric titration had to be carried out by hand, however, it was too laborious a procedure and too insensitive for matching the fast enzyme-catalyzed reactions.

Various pH-stat titrator systems have now become commercially available. These systems make it possible to maintain automatically the reaction pH and to record simultaneously the rate of addition of the titrant. Surprisingly, this method has not been widely adopted for studies of β-lactamase kinetics, being used only by Sabath *et al.* (32), Citri and Zyk (39), and Kuwabara and Abraham (40), and then only partially or in conjunction with other chemical and biological methods.

The authors recently studied the interactions between various penicillins and the extracellular *Bacillus cereus* B569 and *Staphylococcus aureus* Tex-2 β-

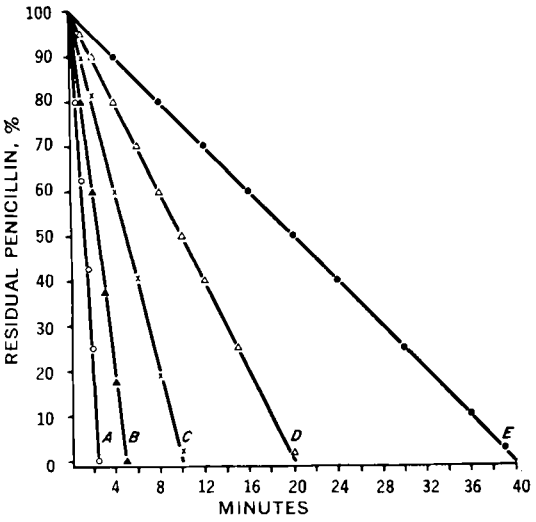



**Figure 1**—B. cereus *B569 β-lactamase-catalyzed inactivation of potassium penicillin G (100 μmoles) at pH 7.0 and 25°. The rate of hydrolysis was shown to be proportional to the enzyme activity (in units): A = 40, B = 20, C = 10, D = 5, and E = 2.5.*

lactamases by a pH-stat alkalimetric titration technique. This method was found to be dependable and usable over a wide range of pH, substrate, and enzyme concentrations. The present paper reports: (*a*) the applicability of the pH-stat alkalimetric titration method to the investigation of β-lactamase kinetics, (*b*) the pH optimums of the β-lactamases from strains of *B. cereus* B569 and *S. aureus* Tex-2, and (*c*) the relative stability of several penicillins toward the staphylococcal β-lactamases. It is hoped that these studies will shed some light on the characteristics of the β-lactamase-catalyzed inactivation of the penicillins.

## EXPERIMENTAL

**Penicillins**—The following penicillins, all of which are either new or established products of the authors' laboratories[1], were used: potassium penicillin G (potassium benzylpenicillin), potassium ampicillin, potassium cyclacillin[2], potassium phenoxymethyl penicillin (potassium penicillin V), potassium phenethicillin [potassium (1-phenoxyethyl)penicillin], 6-(1-aminocyclopentanecarboxamido)penicillanic acid[3] (III), 6-[2-(1,4-cyclohexadien-1-yl)-acetamido]penicillanic acid[4] (IV), 6-(2-amino-2-indancarboxamido)-penicillanic acid[5] (V), 6-(1-amino-3-cyclopentene-1-carboxamido)-penicillanic acid[6] (VI), 6-[2-(1-aminocyclohexyl)acetamido]penicillanic acid[7] (VII), 6-[1-(2-mesitylenesulfonamido)cyclohexanecarboxamido]penicillanic acid[8] (VIII), 6-(2-amino-2-methylpropionamido)-penicillanic acid[9] (IX), and 6-[2-(2-mesitylenesulfonamido)-2-phenylacetamido]penicillanic acid[10] (X). Carbenicillin[11] and 6-[2-amino-(3-trienyl)acetamido]penicillanic acid[12] (XI) were also used.

**Penicillin β-Lactamases**—*S. aureus* Tex-2 β-lactamase was obtained from the supernatant fluid of a fresh broth culture of this organism[13]. *B. cereus* B569 β-lactamase was obtained commercially[14], and it was dissolved in a dilute (0.1%) gelatin A USP solution to make up the desired strength (activity). The enzyme preparation was standardized for each newly prepared sample as described in the *Kinetics* section. No interference due to the presence of gelatin and nutrient broth in the enzyme preparation was observed.

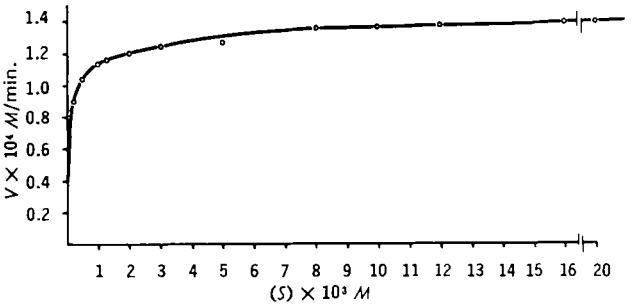



**Figure 2**—*Effect of substrate concentration on the rate of* S. aureus *Tex-2 β-lactamase (2.5 units) inactivation of potassium penicillin G at pH 7.0 and 25°. The initial velocities were plotted against the substrate concentrations.*

**Kinetics**—Rates of hydrolysis were determined by a pH-stat technique with the aid of the Radiometer setup, which is comprised of a PHM26 pH meter, a TTT11 automatic titrator, a TTA3 titration assembly, an SBR2 titragraph, and an ABU12 autoburet (2.5 ml.). The reaction cell was a water-jacketed vessel through which water of the desired temperature (25° except in the effect of temperature studies) was circulated from a constant-temperature bath regulated by a Haacke thermostat (sensitivity ±0.1°). Inserted into the reaction vessel was a glass electrode (G 202B or 202C), a saturated calomel reference electrode (GK2301B), a stirrer, and leads for titrant (0.04 *N* potassium hydroxide solution) and nitrogen gas. The nitrogen gas was washed successively with sulfuric acid (10%), sodium hydroxide (10%) solution, and water before being passed in a slow stream over the surface of the reaction mixture. The proportional band of the Radiometer was set at 0.05.

The substrate (penicillin) solution was prepared fresh and kept cool. A constant volume of the reaction mixture (20 ml.) and a constant ionic strength of 0.025 (for certain reactions ) were maintained by adding a suitable amount of water and potassium chloride. The reaction was initiated by adding the desired amount (about 0.3–0.5 ml.) of the β-lactamase preparation (usually 2.5 units[15]) to the substrate solution in the reaction cell, followed by a trace amount of dilute acid or base or enough to obtain a well-defined starting point.

**Calculations**—The kinetic reaction rate is displayed by the rate of titrant consumption with time under conditions of constant pH. The initial rate (about 30–60% of the entire course of reaction) was usually followed and recorded. The rate $v$, in moles min.$^{-1}$, was calculated from the following equation:

$$v = \frac{N \times 2.5 \times \% \times 0.05}{\text{time in minutes}} \qquad \text{(Eq. 1)}$$

where $N$ is the normality of the titrant (0.04 *N* KOH), 2.5 is the syringe capacity in milliliters, % is the percentage of the course of reaction chosen for calculation (shown on the titration curve), and 0.05 is a mole-molar converting factor.

## RESULTS AND DISCUSSION

**Methodology**—The pH-stat alkalimetric titration method is applicable and dependable for β-lactamase (penicillinase) studies and is particularly useful in obtaining the enzyme kinetic parameters. It is superior to other chemical methods since it: (*a*) promptly determines the activity of the β-lactamase preparation, (*b*) directly and quantitatively measures the β-lactamase susceptibility of the new penicillin from the rate of penicilloic acid formation, (*c*) is devoid of the interference introduced in other methods by reagents

[1] Wyeth Laboratories.
[2] WY-4508.
[3] WY-7953.
[4] WY-12,955.
[5] WY-10,287.
[6] WY-11,912.
[7] WY-14,903.
[8] WY-13,717.
[9] WY-13,687.
[10] WY-13,968.
[11] Beecham Laboratories.
[12] BL-P875, Bristol Laboratories.
[13] Prepared by the Antibiotic Section, Research Division, Wyeth Laboratories.
[14] Riker Co.

[15] The unit of activity of β-lactamase is defined as that amount of enzyme which will hydrolyze 1.0 μmole of potassium penicillin G in 1 min. at 25° and pH 7.0 (buffer free).

Table I—pH Optimum of β-Lactamase Activity as a Function of Substrate, pH, Buffer, and Temperature

| Enzyme Preparation | Substrate | Assay Conditions | pH Optimum | References |
|---|---|---|---|---|
| *S. aureus* Tex-2 (culture supernate) | Penicillin G | pH-stat, 25° | 6.8 | Present work |
| | Phenoxymethyl penicillin | pH-stat, 25° | 6.8 | Present work |
| | Phenethicillin | pH-stat, 25° | 6.8 | Present work |
| | Compound IV | pH-stat, 25° | 6.8 | Present work |
| | Ampicillin | pH-stat, 25° | 6.4 | Present work |
| | Cyclacillin | pH-stat, 25° | 5.8 | Present work |
| | Compound III | pH-stat, 25° | 5.8 | Present work |
| | Ampicillin | pH-stat, 37° | 6.2 | Present work |
| *B. cereus* B569 (purified) | Penicillin G | pH-stat, 25° | 6.4 | Present work |
| | Ampicillin | pH-stat, 25° | 6.2 | Present work |
| *S. aureus* BRL | Penicillin G | Hydroxylamine, 37° | 6.5–7.5 | 42 |
| | 6-APA | 0.5 *M* phosphate buffer | 5.5 | |
| *S. aureus* (culture supernate) | Penicillin G | Iodometric, 30°<br>0.1 *M* sodium citrate<br>0.2 *M* sodium phosphate | 5.8 | 16 |
| *S. aureus* (purified) | Penicillin G | Iodometric, 30° | | 41 |
| | | 0.1 *M* $Na_2HPO_4$–$NaH_2PO_4$ | 5.9 | |
| | | 0.1 *M* tromethamine–HCl | 7.0 | |
| *S. aureus* 147 (purified) | Penicillin G | Iodine–starch, 25° | 6.2 | 43 |
| | 6-APA | Iodine–starch | 7.2 | |
| *B. cereus* NRRL-B569 | Penicillin G | Manometric, 36° | 7.2 | 17 |
| *B. cereus* 569/H | Penicillin G | Hydroxylamine, 37° | 7.0 | 42 |
| | 6-APA | 0.5 *M* phosphate buffer | 5.5 | |

and buffer species, and (*d*) saves time. To study enzyme activity, a standard substrate (*i.e.*, a pure penicillin such as potassium penicillin G) has to be used. To assay the purity of a penicillin, on the other hand, a standard β-lactamase preparation of known activity is required.

Experience has shown that the exact strength of an enzyme preparation is, above all, the most important consideration and that any deviation in its activity or strength during the experiments can lead to serious error. The enzyme preparations used in the present studies were freshly prepared and standardized, and no drastic loss in activity was observed within 3 days if they were kept cold.

**Dependence of Velocity of Reaction on Enzyme or Substrate Concentration**—When reactions were run with different units of β-lactamase and the substrate (penicillin) concentration was held constant, the rate of reaction was shown to be proportional to the enzyme concentration over a wide range. Figure 1 shows that at pH 7.0 and 25°, potassium penicillin G (100 μmoles) was inactivated completely in 2.5, 5, 10, 20, and 40 min. by 40, 20, 10, 5, and 2.5 units, respectively, of *B. cereus* B569 β-lactamase. Under the same conditions, identical results were obtained for the same substrate interacting with the β-lactamase from a strain of *S. aureus* Tex-2. As a matter of fact, the enzyme activity (units) of different enzyme preparations was standardized by using potassium penicillin G as the substrate.

On the other hand, *S. aureus* Tex-2 β-lactamase (2.5 units) interacted with a wide range of concentrations ($2–12 \times 10^{-3}$ *M*) of either potassium penicillin G or ampicillin at pH 7.0 and 25°, giving a hyperbola-type curve when the initial rate was plotted against the substrate concentration. Figure 2 depicts the conventional Michaelis-type plot of penicillin G in which the initial rate varies from first order (at relatively low substrate concentration) to a pseudo-zero-order reaction (at high substrate concentration). At about $10 \times 10^{-3}$ *M*, it seems that virtually all of the enzyme has been saturated with the substrate. From this type of plot, a rather low Michaelis constant ($K_m$) of about 30 μ*M* was obtained for penicillin G, compared with about 700 μ*M* for ampicillin.

**pH–Activity Profile**—The effect of pH on the β-lactamase activity was investigated by allowing 2.5 units of *S. aureus* Tex-2 β-lactamase to interact with 100 μmoles each of penicillin G, phenoxymethyl penicillin, phenethicillin, ampicillin, cyclacillin, and Compounds III, IV, and XI. In each case, a bell-shaped pH–activity profile was obtained, although the pH optimum varied drastically in accordance with the substrate. With penicillin G, phenoxymethyl penicillin, and phenethicillin, which have an aryl or aryloxy side chain, a pH optimum of 6.8 was obtained at 25°.

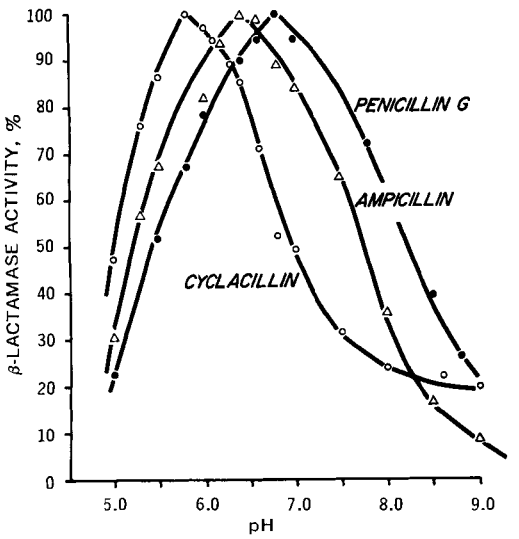



**Figure 3**—S. aureus *Tex-2 β-lactamase (2.5 units) activity as a function of pH and substrate specificity. The pH optimums were shown to be 5.8, 6.4, and 6.8 for the interaction with cyclacillin, ampicillin, and penicillin G, respectively, at 25°.*

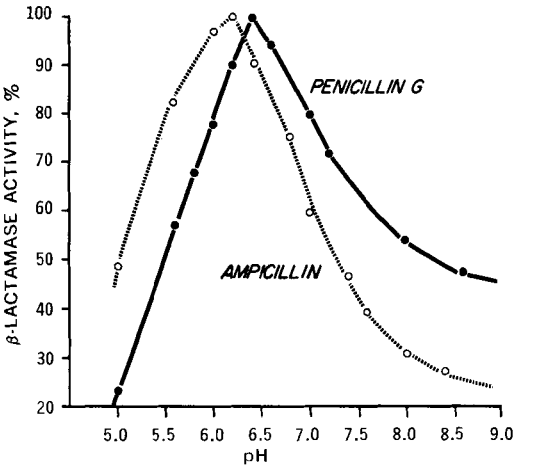



**Figure 4**—B. cereus *B569 β-lactamase activity as a function of pH and substrate specificity. The pH optimums were shown to be 6.2 and 6.4 for interactions with ampicillin and penicillin G, respectively, at 25°.*

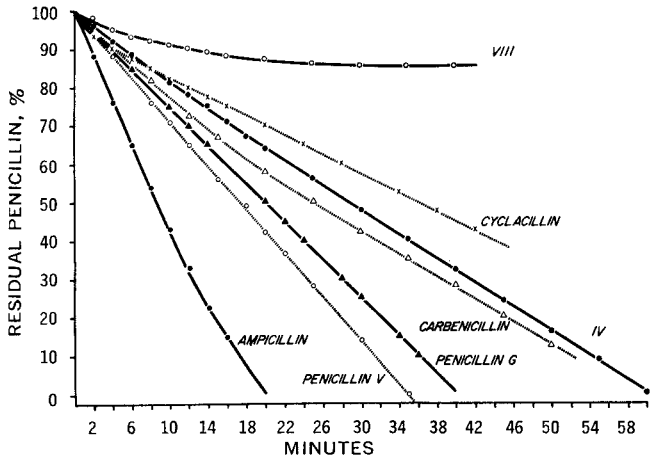



**Figure 5**—*Staphylococcal β-lactamase (2.5 units) inactivation at pH 7.0 and 25° of ampicillin, phenoxymethyl penicillin (penicillin V), penicillin G, carbenicillin, Compound IV, cyclacillin, and Compound VIII (β-lactamase, 5.0 units).*

With ampicillin, on the other hand, which carries a charged (polar) amino group on the aryl side chain, the pH optimum shifted to 6.4, while cyclacillin and Compound III had a pH optimum of 5.8. Figure 3 depicts the pH–activity profiles for the interactions of the *S. aureus* Tex-2 enzymes with the three different types of penicillin.

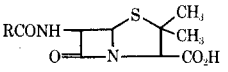
The optimum activity of *B. cereus* B569 was also found to vary with the substrate. As shown in Fig. 4, the pH optimums at 25° were 6.6 and 6.2 when penicillin G and ampicillin, respectively, were the substrates.

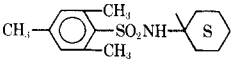
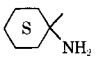
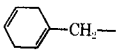
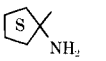
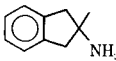
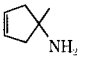
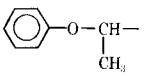
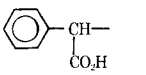
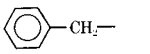
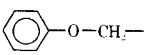
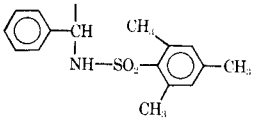
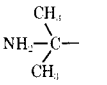
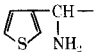
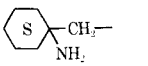
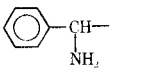
If the pH optimum of a β-lactamase is controlled primarily by the ionization of a key (such as an amino) group of the β-lactamase–substrate complex or simply by an active site of the enzyme, then environmental factors such as temperature, ionic strength, and dielectric constant of the medium must certainly play an important role. The pH optimum for the *S. aureus* Tex-2 β-lactamase-catalyzed hydrolysis of ampicillin was shifted from 6.4 to 6.2 when the temperature was raised from 25 to 37°. On the other hand, Richmond (41) demonstrated that the pH optimum of a β-lactamase varies with the composition of the buffer.

Although earlier studies (7, 8, 20) showed that the pH optimum of a β-lactamase varies with respect to the species of bacteria as well as the enzyme preparation, little was known regarding the substrate dependence. Table I presents the pH optimums obtained in the present studies for several β-lactamases as well as those reported in the literature. The observed pH optimums varied drastically with the substrate, indicating that different types of enzyme–substrate complexes may be formed, depending on the nature of the penicillin side chain. Furthermore, the electronic status and possibly the stereospecificity of the side-chain group of a penicillin molecule may determine not only the rate of hydrolysis but also the particular pH at which the β-lactamase inactivation proceeds at maximum speed.

**β-Lactamase Inactivation of Penicillins *In Vitro* and *In Vivo*—** Despite the drastically different pH optimums of these penicillins, their *in vivo* stability toward β-lactamase should be comparable to their relative *in vitro* stability at neutral pH. As shown in Fig. 5, ampicillin was completely inactivated in about 20 min., penicillin G and phenoxymethyl penicillin in about 36–40 min., and carbenicillin and Compound IV in about 60 min., while cyclacillin was only 50% inactivated in 36 min. by 2.5 units of *S. aureus* Tex-2 β-lactamase at pH 7.0 and 25°. Among the susceptible penicillins investigated under these conditions, ampicillin and Compounds VII, IX, and XI were the least stable, with half-lives ranging from 8 to 12 min. Penicillin G, phenoxymethyl penicillin, phenethicillin, Compounds V and VI, and carbenicillin were intermediate in stability, with half-lives of about 20–25 min. Cyclacillin and Compounds III and IV were the most enzyme-resistant compounds, with half-lives of about 30–35 min., thus being nearly 1.5 times more stable than penicillin G and about four times more stable than ampicillin. Compound VIII, the *N*-sulfonyl derivative of cyclacillin, was about 10 times more stable than the parent compound (cyclacillin), while Compound X, the corresponding derivative of ampicillin, showed no such increase (not shown). The results obtained with *B. cereus* β-lactamase were similar. Table II shows the relative *in vitro* stability (in terms of half-life) of 15 penicillins toward *S. aureus* Tex-2 β-lactamase (2.5 units) at pH 7.0 and 25°.

**Table II**—*Staphylococcus aureus* β-Lactamase Inactivation of Several Penicillins at pH 7.0 and 25°



| Number | Name | Penicillin Side-Chain Structure, R | Approximate Half-Life, min. |
|---|---|---|---|
| 1 | VIII |  | >120 |
| 2 | Cyclacillin |  | 35–36 |
| 3 | IV |  | 30–32 |
| 4 | III |  | 25–26 |
| 5 | V |  | 22–23 |
| 6 | VI |  | 20–21 |
| 7 | Phenethicillin |  | 22 |
| 8 | Carbenicillin |  | 21 |
| 9 | Penicillin G |  | 20 |
| 10 | Phenoxymethyl penicillin |  | 18 |
| 11 | X |  | 12 |
| 12 | IX |  | 11–12 |
| 13 | XI |  | 9–10 |
| 14 | VII |  | 8–9 |
| 15 | Ampicillin |  | 8–9 |

Although the zwitterionic nature of ampicillin, Compound XI, and possibly other α-aminopenicillins gives them their broad antimicrobial activity, it also makes them more susceptible toward the β-lactamases. However, compounds containing an aminoalicyclic side chain directly connected to the penicillin peptide linkage (Compound III and cyclacillin) were more stable toward β-lactamase compared to the α-aminopenicillins. This may be due to the steric effect of the alicyclic side chain which seems to be enhanced by

*N*-substitution as in Compound VIII. In fact, the steric effect has been proven essential for β-lactamase resistance (44). Thus, under the same conditions, the rate of hydrolysis of methicillin, oxacillin, cloxacillin, dicloxacillin, nafcillin, quinacillin, and diphenicillin was too slow to be detectable by a pH-stat method at room temperature, even when the amount of β-lactamase was increased by fivefold.

Although ampicillin (45, 46) and Compound XI (47) are among the most potent, most acid stable, best absorbed orally, and least serum bound of all the broad spectrum β-lactam antibiotics known today, they are both extremely susceptible to penicillinase. The resistance to ampicillin developed by strains of *S. aureus* is invariably associated with the formation of an inactivating β-lactamase (46).

The fact that cyclacillin is more resistant to staphylococcal β-lactamase than ampicillin was previously reported by Rosenman *et al.* (48). In mice, cyclacillin and Compound III were both as effective as nafcillin against a penicillin G-resistant strain of *S. aureus*, while ampicillin and penicillin G were inactive (49). The higher staphylococcal penicillinase stability of cyclacillin and Compound III demonstrated in the present *in vitro* studies correlates well with that previously reported in *in vivo* studies.

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