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Interconversion of Ampicillin and Hetacillin *In Vitro*

GERALD N. LEVY, JOHN V. IOIA, and EDWARD J. KUCHINSKAS[x]

Abstract □ The conversion of hetacillin to ampicillin in aqueous solution was studied by hydroxylamine assay, IR analysis, and ^{14}C-labeling methods. Each technique indicates a half-life of at least 4 hr for hetacillin at physiological pH. The interconversion of ampicillin and hetacillin in aqueous solutions can be controlled by the concentration of added acetone. Excess acetone leads to the formation of hetacillin in aqueous solutions of ampicillin. A scheme for this interconversion is proposed, which accounts for the production of a Schiff base intermediate and penicillenic acid.

Keyphrases □ Hetacillin and ampicillin interconversion in aqueous solution—mechanism, effect of acetone concentration, determination by hydroxylamine assay, IR analysis, and ^{14}C-labeling □ Ampicillin and hetacillin interconversion in aqueous solution—mechanism, effect of acetone concentration, determination by hydroxylamine assay, IR analysis, and ^{14}C-labeling

Ampicillin and hetacillin degrade to different products in aqueous solution. Concentrated solutions of ampicillin form polymers while similar solutions of hetacillin do not produce polymers of the ampicillin type but lead instead to a penicillenic acid with a characteristic UV absorbance peak at 317 nm (1). In a previous report (2), the finding of different degradative pathways for ampicillin and hetacillin in solution indicated that hetacillin could not be rapidly and completely converted to ampicillin and acetone

Table I—Hydroxylamine Color of Penicillins

Compound	A_{515nm}/mg/ml
Potassium penicillin G	0.350
6-Aminopenicillanic acid	0.385
Potassium ampicillin	0.350
Potassium hetacillin	0.170

as had been claimed (3). In the present study, the stability of hetacillin solutions and the rate and manner of appearance of their degradation products were examined. Preliminary studies were reported earlier (2).

EXPERIMENTAL[1]

Ampicillin trihydrate, potassium ampicillin, hetacillin, and potassium hetacillin were obtained from a commercial source[2]. Acetone-2-^{14}C was also obtained commercially[3].

Hydroxylamine assays were performed using the reagents described previously (4). The method was modified by extending the reaction time of the penicillin with neutral hydroxylamine to 20 min before adding ferric ammonium sulfate. This modification produced higher and more consistent color values with the penicillins tested than did the shorter 3-min incubation originally described.

IR determination of intact hetacillin was based on the ratio of absorbances at 1720 (imidazolidinone carbonyl) and 1790 (β-lactam carbonyl) cm^{-1} (5). In this way the amount of intact β-lactam served as an internal standard for the determination of the γ-lactam (imidazolidinone). For this part of the study, 0.1% solutions of hetacillin were prepared in 0.05 *M* K-PO$_4$ buffer (pH 7.4) and subjected to aeration by nitrogen for various times. The solutions were then frozen, lyophilized, and examined as KBr pellets.

Hetacillin labeled with ^{14}C at the 2-position of the imidazolidinone ring was prepared by reacting ampicillin with acetone-2-^{14}C by a modification of the procedure of Hardcastle *et al.* (5). Excess ^{14}C-acetone was removed by washing with nonradioactive acetone and by vacuum drying of the product. The structure of the labeled hetacillin was verified by comparison of the IR spectrum with that obtained from a commercial source[2]. The hydrolysis of ^{14}C-hetacillin was followed by bubbling nitrogen through a 0.1% solution of ^{14}C-hetacillin in 0.05 *M* K-PO$_4$ (pH 7.4). At intervals, a 50-μl aliquot was withdrawn and counted by liquid scintillation spectrometry. Control experiments showed that the amounts of acetone included in the samples had no observable quench. All aging and gas-flushing experiments were conducted at 25°.

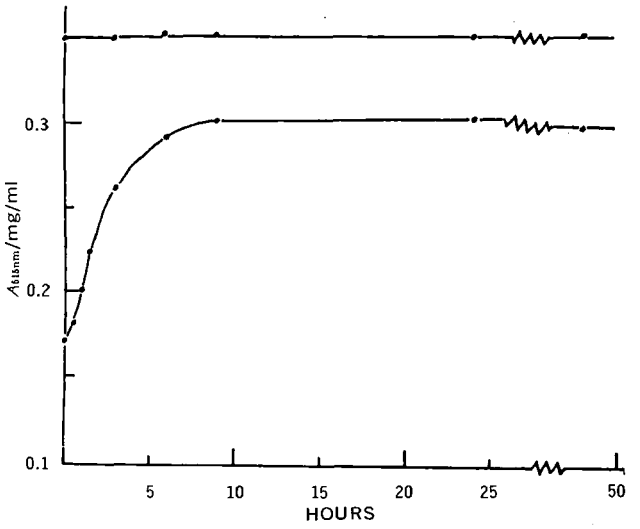


Figure 1—*Development of hydroxylamine color with time. Aqueous solutions of potassium ampicillin (upper curve) and potassium hetacillin (lower curve) in concentrations of 1 mg/ml. Each experimental point is the average of at least three determinations.*

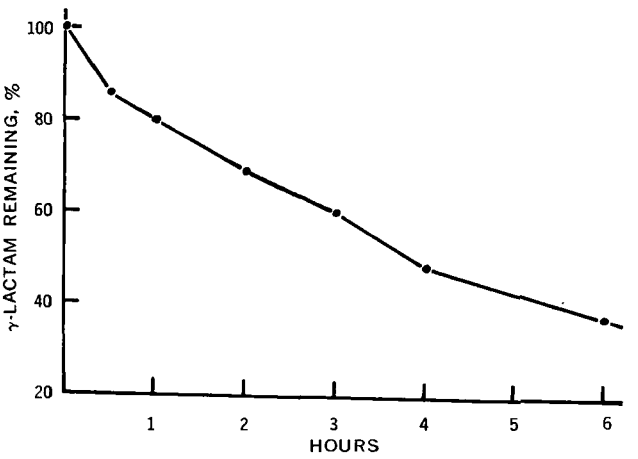


Figure 2—*IR analysis of samples from a hetacillin solution bubbled with nitrogen gas (see* Experimental*). Each data point is a separate flushing experiment for the specified time.*

RESULTS

Stability of Hetacillin—Solutions of hetacillin showed a lower reactivity with hydroxylamine than did solutions of ampicillin, penicillin G, or 6-aminopenicillanic acid. Table I shows that freshly dissolved hetacillin produced less that half the absorbance of other penicillins in the hydroxylamine assay.

When the 0.1% hetacillin solution was allowed to remain at room temperature and aliquots were removed at various times for assay, the color produced increased and approached the ampicillin color value. It was also found that 0.1% ampicillin solutions showed no significant change in their hydroxylamine color value. Figure 1 shows the relationship of the hydroxylamine color value of hetacillin and ampicillin with time.

IR determination of the γ-lactam showed a slow disappearance of 1720-cm^{-1} absorbance. The values for 1720-cm^{-1} absorbance were normalized by taking the ratio of 1720- to 1790-cm^{-1} absorbances. This provides an internal correction for any slight loss of the β-lactam. The decrease in this ratio is shown in Fig. 2.

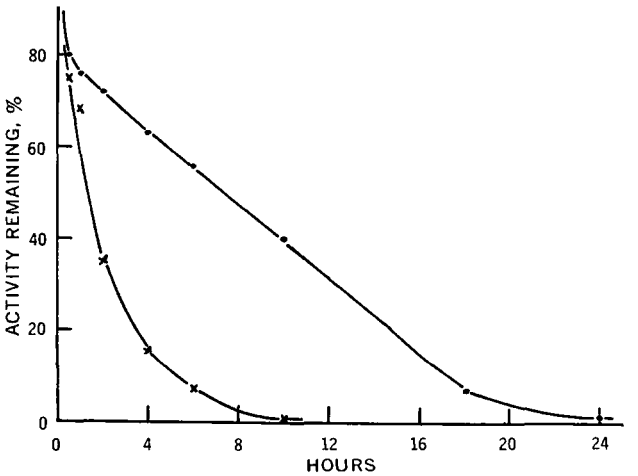


Figure 3—*Comparison of the loss of radioactivity from solutions of ^{14}C-hetacillin (●) and control solutions of ^{14}C-acetone (×) in a stream of nitrogen gas (see* Experimental*). The data shown are typical individual experimental runs performed at least three times.*

[1] IR spectral analyses were performed on KBr pellets using a Perkin-Elmer 21. UV spectra were obtained with a Cary 14, and additional UV measurements were made on a Beckman DU. Radioactivity was determined with a Packard Tri-Carb liquid scintillation counter. NMR spectra were obtained using a Varian A-60 spectrophotometer.

[2] Bristol-Myers Co., International Division.

[3] New England Nuclear.

The loss of radioactivity of ^{14}C-hetacillin is shown in Fig. 3. The volatilization and loss of ^{14}C-acetone from this buffer are also shown as a control. Acetone is removed from the solution far more quickly when free acetone is present than when acetone is chemically combined as hetacillin. The large difference in the loss of radioactivity between solutions of ^{14}C-acetone and ^{14}C-hetacillin indicates that hydrolysis of hetacillin to acetone and ampicillin is not an immediate result of solution.

The results of the three methods used to study the breakdown of hetacillin are compared in Fig. 4. The hydroxylamine method and IR measurements are in close agreement, giving a half-life for hetacillin of 3.5–4 hr. The loss of radioactive acetone was slower than the loss of the γ-lactam. The loss of one-half of the initial acetone required more than 7 hr.

Formation of Penicillenic Acid—A significant difference between solutions of ampicillin and hetacillin is the formation of 317-nm-absorbing material from hetacillin (1). In an attempt to produce UV-absorbing material from ampicillin, various amounts of acetone were added to 10% potassium ampicillin in water. The rate of formation and the final amount of penicillenic acid produced varied with the molar ratio of acetone to ampicillin. The form of this relationship is shown in Fig. 5. In the absence of acetone, ampicillin solutions did not produce any 317-nm absorbance in 24 hr. As the acetone concentration was increased, 317-nm absorption at 24 hr increased. The production of penicillenic acid from ampicillin reached a maximum at a molar ratio of about 4 acetone to 1 ampicillin. At higher concentrations of acetone, this production decreased in both rate and final amount. Similar experiments with hetacillin and acetone showed that adding acetone to 10% aqueous potassium hetacillin resulted in increased production of 317-nm material at 24 hr. Higher concentrations of acetone resulted in a decrease in 317-nm absorbance (Fig. 5).

The time course of the reaction leading to penicillenic acid is illustrated in Fig. 6. The early rate (up to about 2 hr) of production of A_{317nm} material by ampicillin and acetone is less than both the later rate and the rate of production by hetacillin and acetone. In the latter situation, a quick burst of 317-nm-absorbing material appeared within the 1st hr, after which the 317-nm absorbance increased at a somewhat slower rate.

Formation of Hetacillin from Ampicillin in Aqueous Solution—The material formed by incubating ampicillin with acetone in aqueous solution for 48 hr was able to react with neutral hydroxylamine. The amount of 515-nm absorbance produced after 48 hr of incubation was a function of the acetone to ampicillin ratio. Figure 7 shows that as the ratio of acetone to ampicillin was increased, the color value of the solution in the hydroxylamine assay decreased from the value for pure ampicillin to a value (at 20 acetone to 1 ampicillin) that equaled the color value of fresh hetacillin.

A decrease in hydroxylamine assay value could indicate either a loss of the β-lactam or a conversion to a less reactive β-lactam compound. To determine the identity of the material formed from ampicillin and acetone, an aqueous solution of 10% potassium ampicillin with 20 equivalents of acetone was incubated for 48 hr. At the end of the incubation time, the solution was frozen and lyophilized. Samples of the dry powder were examined by IR and NMR and found to give spectra identical to potassium hetacillin. A sample of this material was assayed with neutral hydroxylamine and gave the low extinction of hetacillin. On aging, the color produced by this solution increased to approach the ampicillin value in about 24 hr. Figure 7 shows the increase in color for the hetacillin formed in this way. The rate of conversion of this hetacillin back to ampicillin is very near that shown for hetacillin in Fig. 1.

DISCUSSION

Solutions of hetacillin in water or dilute buffer do not undergo a rapid and complete hydrolysis to ampicillin and acetone. As shown in Fig. 4, the hydrolysis of the γ-lactam of hetacillin has a half-life of nearly 4 hr at an initial concentration of 1 mg/ml and pH 7.4. The loss of IR absorbance characteristic of the γ-lactam carbonyl closely matches an increase in the reactivity of the β-lactam toward neutral hydroxylamine. These results do not distinguish between the conversion of hetacillin to ampicillin or to an intermediate compound. However, the formation of an intermediate with a significant lifespan is indicated by the slow loss of

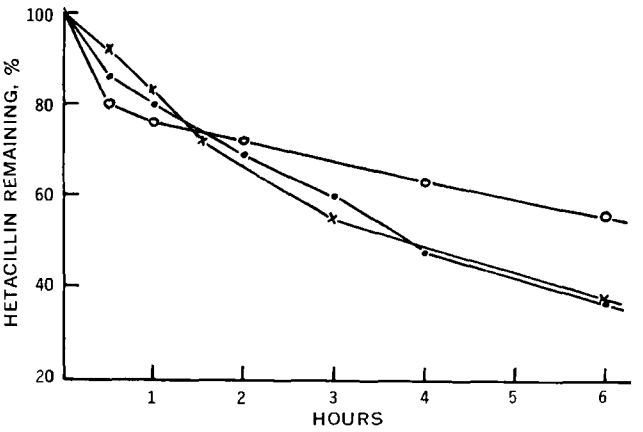


Figure 4—*Correlation of hydroxylamine assay* (×), *IR analysis* (●), *and loss of* ^{14}*C activity* (○) *in terms of remaining hetacillin. (Data derived from experiments of Figs. 1–3.)*

acetone by ^{14}C-hetacillin. The half-life for the loss of the labeled acetone group from ^{14}C-hetacillin is nearly 8 hr.

The intermediate compound formed in the degradation of hetacillin is probably the Schiff base postulated previously (1). This assumption is based on the known participation of the Schiff base in the conversion of free amino compounds to dimethylimidazolidinones (6) and on the direct evidence for the formation of Schiff base in the ampicillin–hetacillin reaction in NMR studies (7).

Since ampicillin does not undergo a penicillenic acid rearrangement to produce 317-nm-absorbing material, the appearance of 317-nm absorbance in solutions of ampicillin with added acetone indicates the presence of either hetacillin or the blocking of ampicillin's free amino group by reaction with acetone as the Schiff base. At high ratios of acetone to either penicillin, the formation of penicillenic acid can be completely blocked. This indicates that it is the Schiff base and not hetacillin that actually undergoes the rearrangement to 317-nm material. If hetacillin degraded directly to the oxazolone compound (penicillenic acid), the presence of excess acetone would have little effect on 317-nm absorbance. However, Figs. 5 and 6 show that excess acetone reduces the formation of penicillenic acid from hetacillin and the formation of penicillenic acid from ampicillin. Figure 6 demonstrates that the rate profiles of penicillenic acid formation differ, depending on whether the starting material is hetacillin or ampicillin.

Beginning with ampicillin and acetone, there is a lag period before the maximum rate of penicillenic acid production is reached. This lag may be accounted for by the formation of the Schiff base intermediate, which can then undergo rearrangement to the 317-nm-absorbing material. In contrast, when the reaction begins with hetacillin or with hetacillin and acetone, the required intermediate is formed by molecular rearrangement rather than by a bimolecular reaction. The more rapid formation of the intermedi-

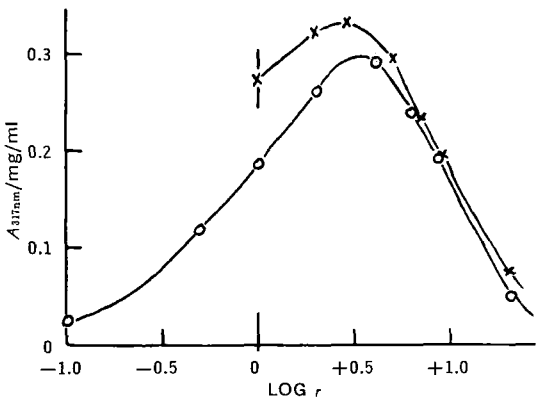


Figure 5—*Effect of the molar ratio* (r) *of* (○) *acetone to 10% potassium ampicillin or of* (×) *acetone to 10% potassium hetacillin on the production of 317-nm absorbance in 24 hr. Each experimental point is the average of at least three determinations.*

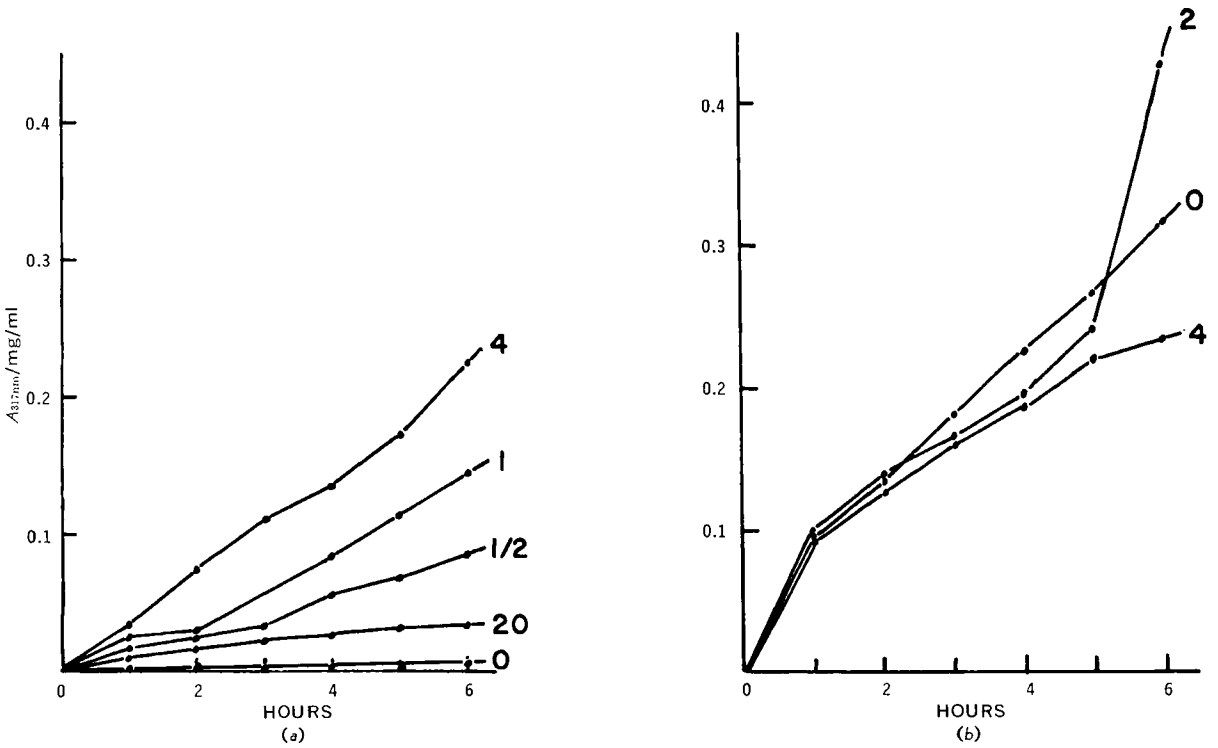


Figure 6—*Production of 317-nm absorbance with time. Key:* (a), *10% aqueous potassium ampicillin plus acetone to give specified molar ratios of acetone to ampicillin; and* (b), *10% aqueous potassium hetacillin plus acetone to give specified molar ratios of acetone to hetacillin.*

ate is reflected in the absence of any lag in penicillenic acid production.

The effect of large excesses of acetone on either hetacillin or ampicillin is to keep the penicillin in a form incapable of forming penicillenic acid. Although low amounts of acetone increase penicillenic acid production, higher amounts drive the reaction beyond the Schiff base intermediate to form hetacillin. The finding that hetacillin forms spontaneously from ampicillin and excess acetone in aqueous solution (Fig. 7) completely supports this proposal.

From the findings presented here, the system shown in Scheme I is proposed. In aqueous solutions of hetacillin, hetacillin and ampicillin are present in an equilibrium as is the Schiff base. A solution of ampicillin alone does not contain any free or potential acetone and cannot form either the Schiff base or penicillenic acid. If the ampicillin solution is concentrated enough, polymers of the type described previously (1) are formed.

The equilibrium between ampicillin, acetone, hetacillin, Schiff base, and water is complicated by the slow removal of material by the irreversible rearrangement of the Schiff base to the penicillenic acid (Step 3). The removal of material by this route would cause the rate of formation of penicillenic acid to be greater when starting with hetacillin than when starting with ampicillin and acetone. The reasoning for this is that the conversion of hetacillin to the Schiff base (Step 2) seems to be more rapid than the conversion of ampicillin plus acetone to the Schiff base (Step

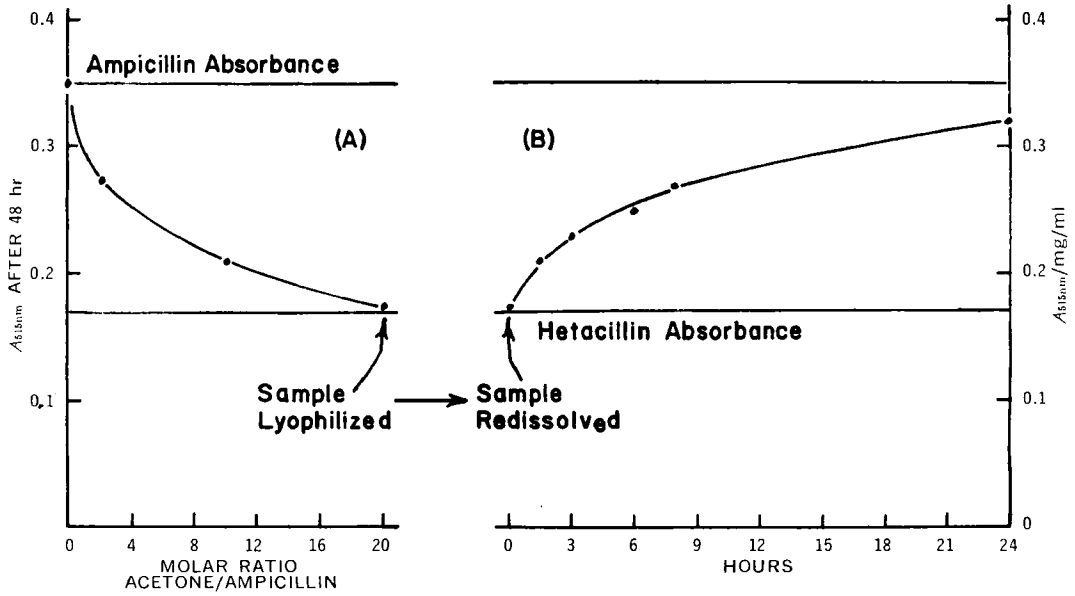
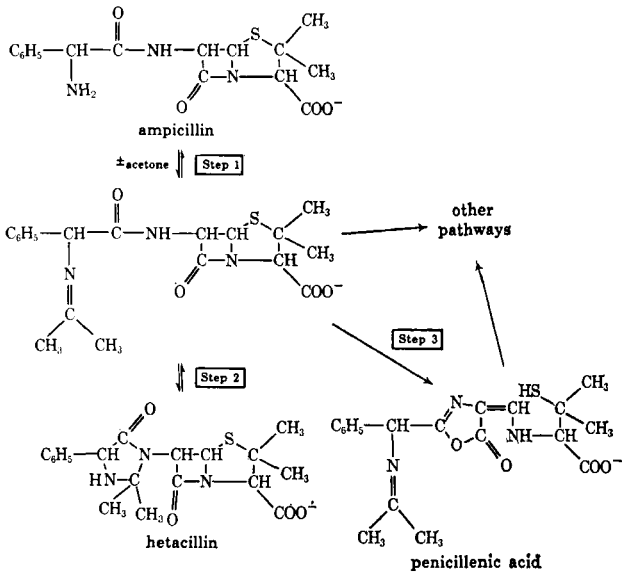


Figure 7—*Hetacillin production and degradation. Key:* (*A*), *production of hetacillin at different molar ratios of acetone and ampicillin in 48 hr reported as absorbances of milligrams per milliliter solution; and* (*B*), *degradation of hetacillin sample formed at a molar ratio of acetone to ampicillin of 20 in 48 hr. Each data point represents the average of at least three separate determinations.*



Scheme I

1). Thus, with hetacillin as the starting material, the Schiff base forms quickly and begins to be removed as penicillenic acid. The slower step of the equilibrium would then be the formation of ampicillin from the Schiff base. With such a mechanism, a definite quantity of penicillin would be lost as penicillenic acid before equilibrium is attained. The preequilibrium buildup of penicillenic acid would be much smaller starting with ampicillin and acetone than with hetacillin.

The effect of high acetone concentrations on ampicillin solutions is readily explained by Scheme I. High acetone drives the reaction to the Schiff base and prevents the back-reaction to ampicillin; moreover, since the Schiff base to hetacillin step is faster than the conversion of the Schiff base to penicillenic acid, hetacillin is the major product. The possible effect of acetone on inhibiting penicillenic acid rearrangement itself needs further study. Additional investigations are also required for an understanding of the factors controlling the conversion of Schiff base to penicillenic acid as well as the possible isolation of this unstable intermediate.

SUMMARY

Solutions of hetacillin in water hydrolyzed to ampicillin and acetone much more slowly than was reported by others. The half-life for the opening of the γ-lactam ring in hetacillin is about 4 hr, while the half-life for the removal of the acetone moiety from hetacillin is nearly 8 hr.

A penicillenic acid with a UV absorbance maximum at 317 nm is produced by solutions of hetacillin, hetacillin with acetone, and ampicillin with acetone. Ampicillin with no acetone present does not produce a 317-nm absorbance. The penicillenic acid is postulated to be derived from a Schiff base intermediate in the reversible interconversion of ampicillin and hetacillin.

These observations and the formation of hetacillin from ampicillin and acetone are accounted for in the proposed scheme. In the scheme neither ampicillin nor hetacillin can go directly to a penicillenic acid but must first form the Schiff base which rearranges to 317-nm-absorbing penicillenic acid. Step 2, the interconversion of the Schiff base and hetacillin, is thought to be faster than Step 1, the interconversion of ampicillin plus acetone and Schiff base. Step 3, the removal of material *via* penicillenic acid, is probably of an intermediate rate.

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