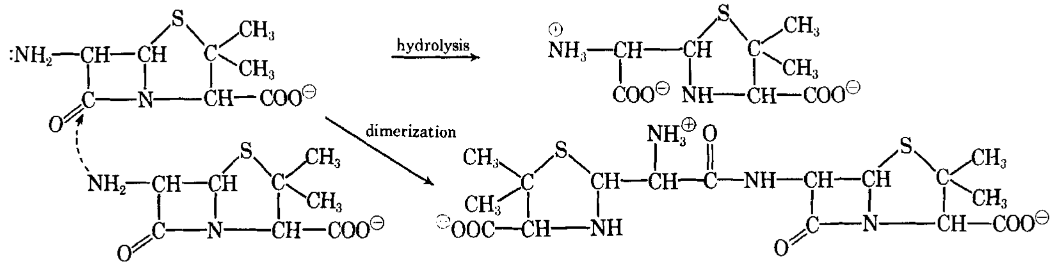
Degradation Kinetics of 6-Aminopenicillanic Acid

By DAVID W. DENNEN

The degradation of 6-aminopenicillanic acid (6APA), as measured by the loss of the β-lactam ring, followed pseudo first-order kinetics with initial concentrations of 0.05–0.92 *M* and in the pH and temperature range of 5.8–6.6 and 35–90°, respectively. Higher orders of degradation rates were approached with increasing pH while the maximum stability of the molecule at all temperatures examined was at approximately pH 8.0. Polymeric materials, measured by ultraviolet absorbance, were formed under all conditions examined.

The finding that solutions of 6APA yielded increasing antibiotic activity to *Staphylococcus aureus* with time was observed in these laboratories (1), and it was suggested that a 6APA dimer was being formed in solution, presumably through nucleophilic attack of the amino group of one molecule to the β-lactam of another to form a penicillin (2). (Scheme I.)



Scheme I

Confirmatory studies remained unpublished when this mechanism was postulated by Grant *et al.*, who isolated a number of antimicrobially inactive polyamides with 7–8 residues from concentrated solutions of 6APA (3). In addition, a material termed factor 1 has subsequently been obtained with antimicrobial activity against *Bacillus subtilis* from 6APA preparations (4). The factor was apparently not an impurity carried with 6APA during purification and appeared to be an antimicrobial condensation product of this molecule containing at least one intact β-lactam ring.

Studies concerned with isolation and subsequent acylation of 6APA for the synthesis of semisynthetic penicillins required an examination of the stability of this molecule. This paper describes the stability of 6APA in terms of its rate of degradation as a function of temperature and pH.

EXPERIMENTAL

Preparation of 6APA—Penicillin nucleus was prepared by the cleavage of the side chain of phenoxymethylpenicillin by amidase (5). The 6APA was then precipitated quickly as the zwitterion at pH 4.7. Repeated solution in base and titration with acid to the isoelectric point produced the product which crystallized as hexagonal plates. The percentage composition of the product was measured as theoretical, m.p. 208–210°, and the pK's 5.5, 4.05 in DMF.

Preparation of Penicic Acid (Open Lactam 6APA)—Penicillinase A (Riker Laboratories) at a concentration of 200,000 units in 5 ml. was suspended within a dialysis tubing in a solution of 6APA (1 mg./ml.) for 24 hr. at 25°. After the 6APA solution gave a negative hydroxamate test, the material was lyophilized. The percentage composition of penicic acid was measured as theoretical and the molecule had pK's 4.7 and 7.8 in water. In 66% acetonitrile, the compound had an R_f of 0.54 and formed a blue color with ninhydrin.

Measurement of β-Lactam Content—Measurement of β-lactam content was performed according to the procedure of Ford (6) by reacting intact β-lactam with neutral hydroxylamine to give the hydroxamate, followed by color development with ferric chloride.

Preparation of 6APA Dimer—6APA solutions (20%) were allowed to stand at room temperature for 1 week, after which they were passed through a G-10 Sephadex column as described in Fig. 6. Hydroxamate positive material eluting between 22–27 ml. was collected and lyophilized. Infrared measurements (Beckman IR-7) revealed the appearance of mono-substituted amide at 6.0 and 6.6 μ and the loss of β-lactam at 5.6–5.7 μ. Titration of the material gave a molecular weight of 430. The

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bulk of the sample moved 5.85 cm. toward the anode during paper strip electrophoresis (pyridine–acetate buffer; pH 4.3, 17 v./cm., 4 hr., 25°) and coincided with the value reported elsewhere for antimicrobially active material derived from 6APA (4). Degradation of the sample at pH 5.8, 75°, yielded an ultraviolet absorbing chromophore with a maximum at 304 mμ.

Degradation of 6APA—6APA was weighed and diluted to volume with preheated buffer of the desired pH. The buffers used were acetate, phosphate, and tris(hydroxymethyl)aminomethane in their respective ranges. The concentration of buffer was systematically twice the molar concentration of the initial concentration (C_0) of 6APA used. The constancy of pH was checked periodically throughout the time studies. No attempt was made otherwise to control the ionic strength to constancy. The buffered 6APA solution was then incubated at constant temperature under nitrogen in order to eliminate access of CO_2 and the possible formation of 8-hydroxypenillic acid (7). Aliquots were taken periodically for various assays described above. Cooling or diluting the aliquots were shown substantially to stop the reaction.

RESULTS

The loss of β-lactam content in 6APA solutions (0.05–0.92 *M*) followed first-order kinetics in the pH range 5.8–6.6 and shifted to second-order rates at pH 7.5–8.0 and above. In Fig. 1, the degradation of β-lactam is plotted in a fashion that designates a second-order reaction as a linear curve, while those data deviating from linearity obey the first-order rate law. Since below pH 7.5, the degradation of 6APA could be described by first-order kinetics at an initial concentration of 0.184 *M*, suggesting simple β-lactam hydrolysis, it was desirable to see if increased initial concentrations of 6APA would lead to second-order rates concomitant with bimolecular polymerization. However, as illustrated in Fig. 2, the first-order rate law was obeyed in increasing initial 6APA concentrations to 0.92 *M*.

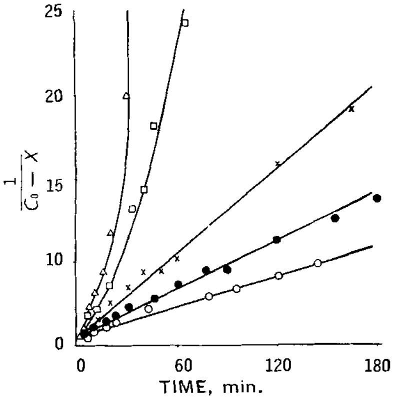

Fig. 1—Rate of β-lactam disappearance at constant initial concentration of 6APA, as a function of pH. First-order curves: pH 5.80 (△), *pH 6.60* (□), *second-order curves: pH 8.50* (×), *pH 8.0* (●), *and pH 7.50* (○). $C_0 - x$ *is the amount of intact β-lactam 6APA. Initial concentrations of 6APA were 0.184* M *and the degradation was measured at 90°.*

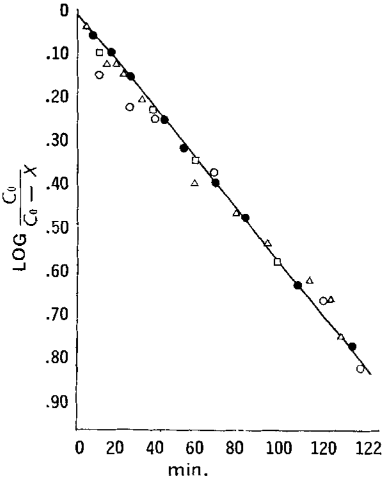

Fig. 2—Rate of β-lactam disappearance with increasing initial concentrations of 6APA. 6APA solutions incubated at pH 6.30, 80°. Initial concentrations: 0.13 M (○), *0.365* M (□), *0.42* M (●), *0.92* M (△). C_0 *is the initial concentration of 6APA, and* $C_0 - x$ *is the amount of intact β-lactam.*

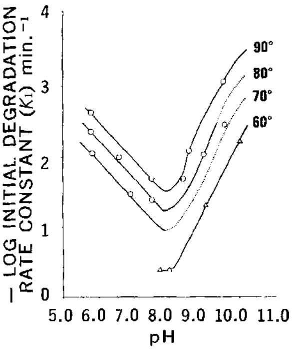

Fig. 3—Initial rate of β-lactam loss in 6APA solutions. Initial rate constants determined in 6-APA solutions by the rate of hydroxamate disappearance (○) *and by titration in a pH-stat* (△).

An examination of 6APA degradation at various pH's and temperatures revealed maximum stability of this molecule at approximately pH 8.0, well above the pKa (5.5) of its free amino group. In Fig. 3, the stability of 6APA β-lactam is depicted in relation to the initial rates of degradation. The data presented were obtained by two methods: disappearance of the 6APA reactivity to hydroxamate, and electrometric titration of the proton liberated by the opened β-lactam. It is important to note that in the degradation reaction below pH 8.0, the free amino group shifts from pKa 5.5 to that characteristic of an α-amino group and no base is consumed in the pH-stat titration.

The initial rates of degradation of β-lactam at an initial concentration of 0.184 *M* are plotted according to the method of Arrhenius in Fig. 4 as a function of temperature. The activation energy (E_a) for the degradation was calculated to be 17 Kcal./mole, comparing with the inactivation of phenethicillin (8), for example, at 17.6 Kcal./mole and benzylpenicillin (9) at 20 Kcal./mole. The activation energy associated with these latter penicillins can be essentially attributed to the formation of penicilloates (10) (β-lactam hydrolysis) at those pH's examined. The energy associated with 6APA degradation, however, must take into account that activation energy pre-empting any polymerization that occurred simultaneously with β-lactam hydrolysis. Since second-order kinetics were visualized only in alkaline solutions, the distinct possibility was presented that penicic acid, formed initially from 6APA, could in turn prime the polymerization of 6APA. Below pH 7.5 it could not participate as a nucleophile, and 6APA would degrade simply through β-lactam hydrolysis as observed kinetically. The consequence of such a polymerization mechanism, however, would preclude the retention of an intact β-lactam in the dimer or polymer formed to account for the observed increase in antimicrobial activity.

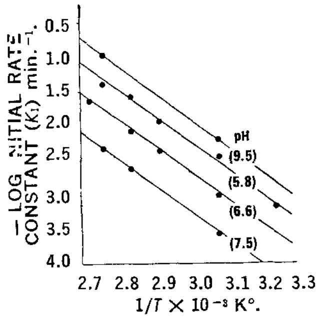

Fig. 4—Effect of temperature on the initial rate loss of β-lactam as a function of pH. Rate constants determined with 0.184 M *solutions of 6-APA.*

Since solutions of 6APA are not routinely allowed to remain at pH's much above neutrality, it was of importance to examine the character of the degradation product at pH's below 7.0 rather than in alkaline solution in order to determine whether polymerization was occurring at a rate proportional to the first rather than second order of the remaining 6APA concentration.

To measure polymerization, advantage was taken of an ultraviolet absorbing material that appeared during the progress of 6APA degradation. The ultraviolet spectra of this material shown in Fig. 5, had a maxima at 304 mμ while penicic acid, when similarly degraded from nearly the same initial concentration, had very little absorbance at this wavelength.

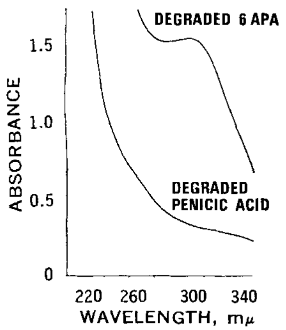

Fig. 5—Ultra violet spectra of degraded 6APA and penicic acid. Initial 6APA concentration, 2.5%; initial penicic acid concentration, 2.0%. Degradation for 2.5 hr., 75°. Degradation mixtures diluted 1:-12.5 for assay.

A degradation mixture of 6APA containing this chromophoric material was passed through a G-10 Sephadex column, and as shown in Fig. 6, it is clear that this material represented a molecular size greater than 6APA. Species were present in the effluent which coincided in position to phenoxymethylpenicillin and benzylpenicillin while the leading edge of the effluent yielded materials of greater molecular size than the penicillins. Any condensation that could occur with 6APA or penicic acid to form diketopiperazines did not seem to involve an increase in the absorbance at 304 mμ. The 304 mμ chromophore thus appeared to represent subsequent rearrangement of 6APA to penaldehyde and/or azlactone-type compounds following the acylation of one molecule by another.

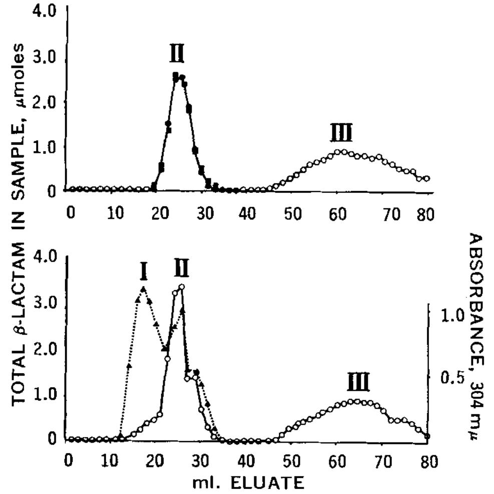

Fig. 6—Separation of penicillins, 6APA, and 6APA degradation mixture by gel-filtration. Top: 14.9 μmoles penicillin and 11.50 μmoles 6APA applied to column, phenoxymethylpenicillin (■), benzylpenicillin (●), 6APA (○). Recovery of total β-lactam, 88%. Bottom: the starting material was 6APA which was degraded and then applied to the column. Intact β-lactam of 6APA and polymeric material (○), 304 mμ absorbing material (▲). Sephadex G-10 column 1 × 39 cm. with 39 cm. head of buffer for elution. Peaks I, II, *and* III *represent polymeric material, penicillin, or 6APA dimer and 6APA, respectively.*

In Fig. 7, the formation of the 304 mμ absorbing material is shown in degradation mixtures of 6APA at an initial concentration of 0.184 *M* as a function of temperature at pH 6.30. It is apparent that a constant amount of polymeric material, represented by ultraviolet absorbance, was formed at this initial concentration. The addition of varying amounts of penicic acid to 6APA failed to increase the 304 mμ absorbance yield, and thus it appeared that the former was not necessary to prime the polymerization. It is also interesting to note that the total polymeric material produced from increasing initial concentrations of 6APA did not increase as the square of the concentration, but rather was directly proportional to the initial concentration of 6APA as shown in Fig. 8. Furthermore, the formation of polymeric materials adjudged from the 304 mμ absorbance in 6APA degradation reactions at pH 6.3 was confirmed at pH 5.8 and 6.0 as well, indicating that polymerization occurred as a parallel reaction to 6APA hydrolysis.

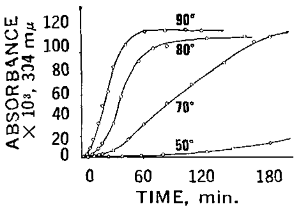

Fig. 7—Formation of 304 mμ absorbing material from degraded 6APA solutions. Initial 6APA concentration, 0.184 M, pH 6.30.

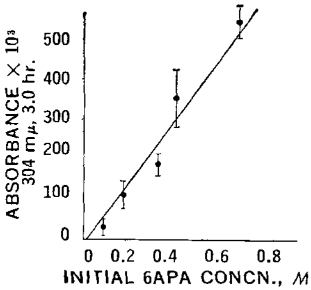

Fig. 8—Maximum level of 304 mμ absorbance as a function of the initial 6APA concentration. 6-APA degraded at pH 6.30, 80°.

DISCUSSION

The rates of β-lactam hydrolysis and dimerization of 6APA are both proportional to the first order of the intact β-lactam content in solutions below pH 7.5. This is not a surprising result since no direct measure of 6APA monomeric concentration is obtained by the assay of intact β-lactam in a system undergoing hydrolysis and polymerization. The dimerization or further polymerization of 6APA involving a β-lactam acylation by another 6APA molecule always leaves one β-lactam intact per two molecules of 6APA. The rate of dimerization would thus be proportional to the square of the 6APA concentration, whereas it would be directly proportional to the first-order loss of β-lactam.

Since the activation energy (E_a) for the hydrolysis of other penicillins is similar to that measured in the degradation of 6APA, it is reasonable to conclude that both β-lactam hydrolysis and intermolecular acylation occur with equal ease. This is one suggested explanation for the proportionately direct increase in the amount of polymer formed as a function of the initial 6APA concentration. Otherwise, one might expect that the rate of dimerization, which is proportional to the square of the initial concentration of 6APA, would be reflected by a proportionate increase in the polymer yield.

In alkaline solution, second-order kinetics of β-lactam loss are apparent from the data, and in these instances the β-lactam content can be visualized to closely approach the actual remaining concentration of 6APA. The increased lability of an acylated β-lactam over that of 6APA affects the apparent loss of two β-lactams per dimer rather than one.

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Cytotoxicity of Rubber Closures in Tissue Culture Systems

By P. J. VASINGTON, H. D. PIERSMA, J. J. CORBETT, and J. L. BITTLE

Rubber closures used in the pharmaceutical industry were tested for cytotoxicity in primary cultures of chick embryo cells and in cultures of cells passaged from human malignant tissue. Assay was by three methods: direct contact, growth-extract, and maintenance-extract. In the first, rubber samples were placed in direct contact with cell monolayers. In the growth-extract system, a sample was incubated in growth medium, and the resultant extract was placed in a cell suspension. In the maintenance-extract method, following incubation of a sample in maintenance medium, the extract was overlayed on a cell monolayer. Tissue culture provided a more sensitive test of toxicity in comparison with the generally accepted implantation of rubber in the paravertebral muscles of rabbits.

Testing the toxicity of rubber closures for pharmaceutical applications has been primarily physicochemical. To date, there is no acceptable biological assay system, although the rabbit implantation method of Brewer and Bryant (1) for screening plastics has gained considerable favor.

The need for a rapid, economical, and reproducible *in vitro* test has led to the investigation of

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