Fluorometric Analysis of Ampicillin in Biological Fluids

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Abstract □ Ampicillin forms a strongly fluorescent yellow product in acid solution during hydrolysis at elevated temperature. The quantity and rate of formation of the fluorescent material are enhanced appreciably by formaldehyde, and the fluorescent product exhibits extraction properties of an organic acid. In alkaline solution, the fluorescent compound has uncorrected excitation and emission maxima at 346 and 422 nm., respectively. An assay procedure based on these observations permits detection of less than 0.05 mcg./ml. of ampicillin and/or α-aminobenzylpenicilloic acid. By combining fluorometric and microbiological methods, measurement can be made of ampicillin degradation products as well as the unchanged antibiotic in serum and urine.

Keyphrases □ Ampicillin analysis—biological fluids □ Biological fluids—ampicillin and degradation product, analysis □ pH effect—ampicillin product fluorescence □ Hydrolysis products—ampicillin □ TLC—separation □ Microbiological—fluorometric analysis—ampicillin □ Fluorometry—analysis

Several methods for the quantitative measurement of ampicillin (α-aminobenzylpenicillin) in aqueous solution have been described. These utilize techniques such as UV spectrophotometry (1), iodine absorption (2), and hydroxamate color formation (3). Recently, Smith *et al.* (4) described a spectrophotometric assay method based on the hydrolysis of ampicillin to α-aminobenzylpenicillenic acid which exhibits absorption at 322 nm. Because of its relative insensitivity, this procedure is unsuitable for measurement of ampicillin at low concentrations in biological fluids. To date, procedures based on microbiological assay (5, 6) have been the only nonisotopic methods suitable for detection of ampicillin, as well as many other penicillins, at the low concentrations encountered in serum and urine following therapeutic doses of the drug.

During the investigation of the spectrophotometric method of Smith *et al.* (4), it was found that a strongly fluorescent product could also be formed from ampicillin. This observation led to the development of a highly sensitive fluorometric method for quantitative measurement of ampicillin and at least one of its degradation products in serum and urine.

EXPERIMENTAL

Apparatus—An Aminco-Bowman spectrophotofluorometer was employed for measurement of fluorescence. A Beckman Expandomatic SS-2 pH meter and a Beckman DB spectrophotometer were used for pH and UV absorption determinations, respectively. Electrophoresis was performed in agar gel using a Laurell-type electrophoresis apparatus[1].

Materials—Analytical grade sodium ampicillin[2] (potency: 865 mcg./mg.) was used as the reference material for all studies. Stock solutions containing 100 mcg./ml. of ampicillin (corrected for potency) were either prepared fresh or were stored frozen and used within 5 days. Potassium hetacillin[2], potassium benzylpenicillin[3], and cephalexin[4] were analytical grade materials provided by the manufacturers. Methicillin, oxacillin, cephalothin, and ampicillin powder for injection were used by dilution of the contents of proprietary vials of parenteral material.

Buffers—Measurements carried out at specific pH values involved use of the following buffer systems: at pH < 2, HCl–KCl (7); at pH 2–8, citric acid–disodium phosphate (2); at pH 8–11, disodium phosphate–NaOH (7); and at pH > 11, KCl–NaOH (7). All pH values were obtained at 20° at the time of use of the buffered solutions.

Effect of pH on Ampicillin Product Fluorescence—A solution of the fluorescent product was obtained by heating 100 mcg. of ampicillin in 40 ml. of pH 3.4 citrate–phosphate buffer for 2 hr. at 75°. One-milliliter aliquots of this stock solution were mixed with 15 ml. of each of 15 buffers which covered the pH range of 0–14. The fluorescence intensity and final pH of each solution were then measured.

Effect of pH on Formation of Product—One milliliter containing 100 mcg. ampicillin was added to 39 ml. of various buffers (pH range 1–10) which were preheated to 75°. At 30-min. intervals, 1-ml. aliquots were withdrawn from each of the 11 solutions and added to 2 ml. of 2 *N* NaOH. Fluorescence readings were obtained until increases in intensity were no longer apparent. The study was also repeated at a higher temperature in the buffer system that was found to yield maximum fluorescence (pH 2). Solutions containing 0.25 *M* citrate buffer and 5 mcg./ml. of either ampicillin or hetacillin were heated at 90° in the presence and absence of 0.8% formaldehyde. Aliquots of these mixtures were removed for fluorescence measurements at various times up to 5 hr.

Solvent Extraction—Spectroscopic grade organic solvents were used to determine whether ampicillin or the fluorescent product could be extracted from aqueous solution. The solvents tested were: acetone–chloroform (1:1), chloroform, ethylene dichloride, hexane, heptane, and carbon tetrachloride. Known concentrations of ampicillin or its fluorescent product were placed in 5 ml. solution buffered to pH 1, 2, 3.4, or 7 and shaken with an equal volume of organic solvent. After agitation for 1 hr., fluorometric measurement of the concentration of drug or product remaining in the aqueous phase was made.

Other Assay Methods—Ampicillin in aqueous solutions at a concentration of about 500 mcg./ml. was quantitated by UV spectrophotometry (1). In particular, the contents of four vials of ampicillin for injection were diluted with water and the absorbance measured at a wavelength of 258 nm.

A microbiological assay was employed along with the fluorometric method to measure ampicillin in aqueous solutions and in biological samples at concentrations less than 5 mcg./ml. The agar-well diffusion assay described by Bennett *et al.* (5) was used, employing *Bacillus subtilis*[5] as the microorganism.

Biological Samples—Serum and urine specimens were obtained after intravenous administration of 570 mg. ampicillin to a normal adult male subject (85 kg. weight). In addition, urine samples were collected for 36 hr. after oral administration of 500 mg. ampicillin to the same subject. All samples were refrigerated and analyzed within 3 days.

Electrophoresis—Serum samples, which were obtained after intravenous administration of ampicillin were subjected to agar gel electrophoresis. Aliquots of 0.01 ml. of serum were applied to the agar gel which was buffered with tris–maleate at pH 5.6 (8) Electrophoresis of these, as well as ampicillin standards, was carried out at approximately 240 v. and 60 ma. for 45 min. The agar gel was then overlayed with 1.5% nutrient agar[5] containing *B. subtilis* spores. After incubation at 35° for 16–20 hr., the bioautogram was examined for zones of bacterial inhibition.

[1] Metaloglass Inc., Boston, Mass.
[2] Bristol Laboratories, Syracuse, N. Y.
[3] Chas. Pfizer & Co., Brooklyn, N. Y.
[4] Eli Lilly and Co., Indianapolis, Ind.
[5] Difco Laboratories, Detroit, Mich.

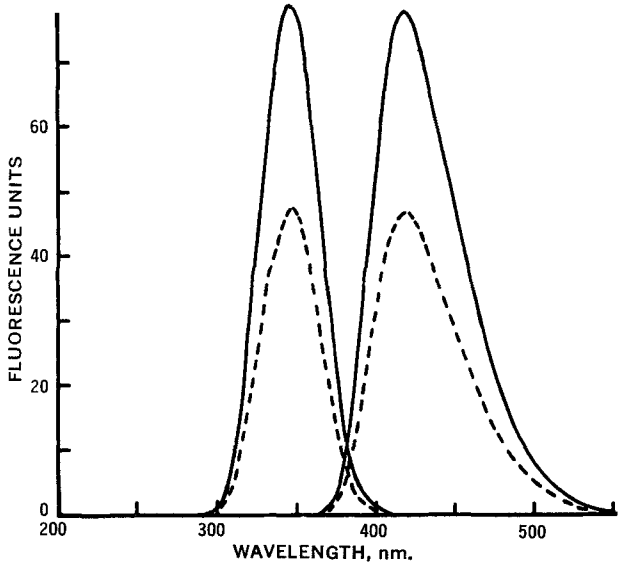


Figure 1—*Spectrophotofluorometric excitation (346 nm.) and emission (422 nm.) spectra for the fluorescent product of ampicillin reference material* (——) *and from urine* (- - -).

Chromatography—A composite sample of urine collected after oral administration of ampicillin was diluted, buffered to pH 3.4, and heated at 75° for 2 hr. Extracts of the fluorescent product in 4 ml. of acetone–chloroform or 4 ml. of chloroform were then used to initiate 16-tube countercurrent distribution (CCD) studies. The aqueous phase consisted of 6 ml. of pH 3.4 citrate–phosphate buffer. At the completion of 15 transfers, 1 ml. of 10 *N* NaOH was added to each tube. Following equilibration, an aliquot of the aqueous phase was fluorometrically analyzed. The method of Williamson and Craig (9) was then used to generate calculated distribution profiles for the two CCD systems.

Similar acetone–chloroform extracts of fluorescent product from ampicillin in urine and from the reference material were subjected to TLC on Eastman silica gel sheets. The solvent systems used included: A, chloroform–acetone (1:1), B, 1-butanol–methanol (4:6), and C, ethylene dichloride–dioxane–water (2:1:1).

Ampicillin Conjugates—Test for the presence of glucuronide or sulfate metabolites of ampicillin was carried out on composite urine samples obtained after oral dosage of the drug. Two milliliters of urine was mixed with 1 ml. of 0.4 *M* pH 5.2 acetate buffer and 1 ml. of either glusulase[6], containing 18,000 units of glucuronidase and 5000 units of sulfatase, or Ketodase[7], containing 5000 units of β-glucuronidase. Samples with and without enzyme were incubated at 35° for 20 hr. and then assayed microbiologically.

Effect of Penicillinase—A degradation product of ampicillin, α-aminobenzylpenicilloic acid, was formed in aqueous solution by hydrolysis of ampicillin with penicillinase[8]. A solution containing 20 mcg./ml. ampicillin and 80 kinetic units/ml. of the enzyme in *M*/15 pH 7.4 Sorensen's buffer was incubated at 37° for 1 hr. At various intervals, 1 ml. of solution was removed and added to 3 ml. of 10% trichloroacetic acid maintained at 0°. One portion of this sample was analyzed by the fluorometric method while another portion was neutralized and assayed microbiologically.

RESULTS

Formation and Properties of the Ampicillin Product—When ampicillin is subjected to heating in acid solution, a strongly fluorescent yellow product is formed. The absorption and emission spectra of this product are shown in Fig. 1. The fluorescence profiles for products formed from analytical grade ampicillin and from ampicillin excreted in urine are identical. All fluorescence measurements in this study were obtained at the excitation maximum at 346 nm. and the emission maximum at 422 nm. (uncorrected).

[6] Endo Laboratories, Garden City, N. Y.
[7] Warner-Chilcott, Morris Plains, N. J.
[8] Bioquest Laboratories, Cockeysville, Md.

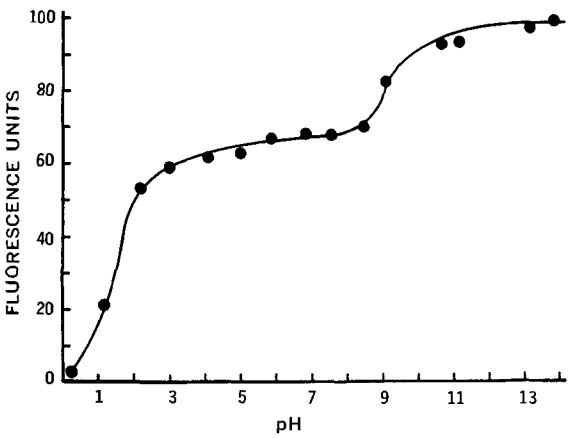


Figure 2—*Relative fluorescence intensity of equal concentrations of the ampicillin product in solutions of various pH values.*

Measurements of the fluorescence intensity of equal concentrations of the ampicillin product in solutions at various pH values yielded the results shown in Fig. 2. The fluorescence of the ampicillin product is greatest at pH values greater than 12. All measurements of ampicillin product fluorescence were, therefore, usually obtained in 1 *N* NaOH solution. The pH–fluorescence profile is an indication that the ampicillin product has at least two ionization constants, namely between pKa 1 and 2 and between pKa 9 and 10.

The effect of pH on the relative amount of fluorescent product formed from ampicillin after heating for 4 hr. at 75° is shown in Fig. 3. An appreciable quantity of product is generated in acid medium, with the maximum amount formed over the pH range of 2–3. Practically no fluorescent material is produced in solutions at pH values greater than 7. The rate of formation of the fluorescent product at 90° in pH 2 buffer in the presence and absence of formaldehyde is shown in Fig. 4. Formaldehyde exerts a catalytic effect on the reaction, causing both an increased rate of formation of the product and an increased total amount of the material. A similar experiment with hetacillin, which is known to hydrolyze rapidly in aqueous solution to ampicillin (10), produced essentially identical results.

The extractibility of the fluorescent product from acid media was tested with several organic solvents. The compound is readily extracted into acetone–chloroform (1:1), chloroform, and ethylene dichloride but is practially insoluble in hexane, heptane, and carbon tetrachloride. From either acetone–chloroform or chloroform, the product can be subsequently partitioned into 1 *N* NaOH. Ampicillin itself, on the other hand, could not be extracted from acidic solution into any of the solvents tested.

At ampicillin concentrations less than 50 mcg./ml., only a single fluorescent product appears to be formed. This was apparent from TLC of acetone–chloroform extracts of the reaction mixture. In the solvent systems used, single fluorescent spots were found with R_f values of 0.80 (A), 0.74 (B), and 0.62 (C). However, at appreciably higher ampicillin concentrations, TLC revealed multiple fluorescent products with R_f values less than that of the primary product These may have been the result of some polymerization since the

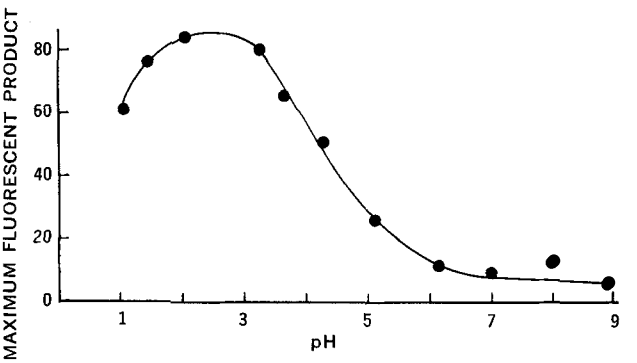


Figure 3—*Effect of pH on the maximum amount of fluorescent product formed from 2.5 mcg./ml ampicillin at 75°.*

1. *Nonprotein solution:* mix 1 ml. of solution with 1 ml. of *M*/2 pH 2 citrate buffer.

1*a*. *Protein solution:* mix 0.5 ml. of sample with 2 ml. of 10% trichloroacetic acid. Centrifuge to obtain 2.0 ml. of clear supernatant.

2. Add 0.5 ml. of 7% formaldehyde in 0.4 *M* pH 2 citrate buffer.
3. Heat solutions in water bath at 90° for 2 hr. and then cool to room temperature.
4. *Direct:* add 1 ml. of 2 *N* NaOH to each solution.

4*a*. *Extraction:* add 7 ml. of acetone–chloroform (1:1). Shake 30 min.

4*b*. Shake 5 ml. of organic phase with 2 ml. of 1 *N* NaOH for 10 min.

5. Measure fluorescence of alkaline solutions at 346-nm. excitation and 422-nm. emission wavelengths.
6. Reference standards of ampicillin should be simultaneously included.

Scheme I—Procedure for fluorometric assay of ampicillin

fluorescence profile showed an additional absorption peak at 285 nm. (1).

Proposed Method—The procedures evolved for the fluorometric analysis of ampicillin in biological and nonbiological fluids are outlined in Scheme I. The method is based on the observations outlined and is useful for ampicillin concentrations as high as 20 mcg./ml. and as low as 0.01 mcg./ml. When very low concentrations of ampicillin are being measured, reference standards should include a drug-free blank from each individual studied. This ensures against patient variation in the fluorometric blank characteristics of samples containing proteins.

Dosage Form Analysis—Microbiological, spectrophotometric, and fluorometric methods of analysis were used to determine the ampicillin content of four proprietary vials of injectible sodium ampicillin. The results of these determinations are shown in Table I. A chi-square analysis of the data ($\chi^2 = 1.26$ on 6 *df*) showed that all techniques gave comparable results.

Other Antibiotics—The assay procedure was tested, using several penicillin or cephalosporin derivatives as shown in Table II. As expected (10), hetacillin forms, on an equimolar basis, as much of the fluorescent product as does ampicillin. Methicillin, oxacillin, and cephalothin formed no detectable fluorescence. Of particular interest is that benzylpenicillin, which lacks the α-amino group of ampicillin, forms no fluorescent product. Cephalexin, however, yielded a small amount of the fluorescent material. This cephalosporin derivative has the α-aminobenzyl group as does ampicillin, but the β-lactam ring is more resistant to acid hydrolysis. These data show that the aminobenzyl group and a cleaved β-lactam ring are necessary for the formation of the fluorescent product.

Biological Sample Analysis—Known concentrations of ampicillin in aqueous solution and added to pooled human plasma were subjected to the fluorometric assay procedure without the organic extraction steps. Over the range of 0.2–10 mcg./ml., there was a linear relationship between ampicillin concentration and fluorometer response, as shown in Fig. 5. The relative recovery of the antibiotic from the protein solutions compared to the aqueous solutions was 99.6 ± 5.2%. Thus, ampicillin can be quantitatively recovered from plasma using trichloroacetic acid as the protein precipitant.

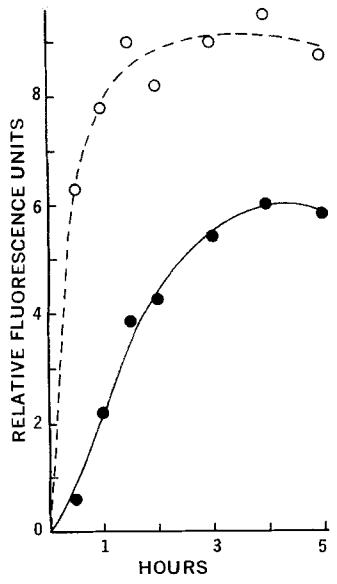


Figure 4—*Rate of formation of the fluorescent product from 2.5 mcg./ml. ampicillin at 90° in the presence* (○) *and absence* (●) *of 0.8% formaldehyde.*

Table I—Comparison of Three Assay Methods: Recovery of Ampicillin from Commercial Vials of Injectable Sodium Ampicillin

Product	Labeled Contents, mg.	Percent of Label Claim[a] Microbiological	UV	Fluorometric
1	1000	112	114	118
2	500	122	118	127
3	500	125	107	114
4	500	110	107	116

[a] Chi-square test: 1.26 on 6 *df*.

Serum samples, obtained after intravenous administration of 570 mg. ampicillin to a normal male subject, were analyzed for ampicillin by the fluorometric and microbiological methods. The two methods yielded some appreciable differences (Fig. 6), particularly in samples removed during the later part of the collection period. Such differences are characteristic of the presence of a metabolite or degradation product in the samples. The specificity of the microbiological assay was checked by agar gel electrophoresis and bioautography. Unchanged ampicillin was found to be the only substance in plasma that inhibited the growth of the test organism.

Urine samples were also collected after ampicillin administration, and the fluorometric and microbiological assays were applied. Differences in results obtained by the two methods were similar to those noted in serum. Enzymatic treatment of composite urine samples with β-glucuronidase and/or sulfatase yielded no change in the quantity of microbiologically active ampicillin. Thus, the metabolite of ampicillin does not appear to be a glucuronide or sulfate conjugate.

The fluorescent product obtained from ampicillin excreted in urine was subjected to two CCD systems. The aqueous/organic distribution coefficient of the ampicillin product was found to be 0.18 in the acetone–chloroform system and 0.43 in the chloroform system. To calculate the expected CCD profiles for a single solute, these values were used with the mathematical relationships described by Williamson and Craig (9). The calculated data and the experimental measurements are shown in Fig. 7 for the two CCD systems. The relatively good agreement between the calculated lines and the data points suggests strongly that a single fluorescent product is formed from ampicillin and its metabolites in urine when present in low concentrations. This observation was further confirmed by TLC of an acetone–chloroform extract of the fluorescent product from ampicillin excreted in urine and ampicillin reference material.

Products of Hydrolysis—Two possible degradation products of ampicillin are its penicillenic acid and its penicilloic acid derivatives. The present assay method utilizes hydrolytic conditions similar to those employed by Smith *et al.* (4), who were able to form and measure *in vitro* the penicillenic acid product of ampicillin. At the low ampicillin concentrations encountered in the biological samples collected in the present study, it was not possible to detect penicillenic acid absorbance at 322 nm. before or after acid hydrolysis.

Table II—Relative Amount of Fluorescent Material Formed from Various Penicillin or Cephalosporin Derivatives

Antibiotic	Concentration, mcg./ml.	Percent of Ampicillin Fluorescence
Ampicillin	1,3,5	100
Hetacillin	1,3,5	102 ± 7
Benzylpenicillin	5	0
Methicillin	~5	0
Oxacillin	~5	0
Cephalexin	1,3,5	8.9 ± 0.4
Cephalothin	~5	0

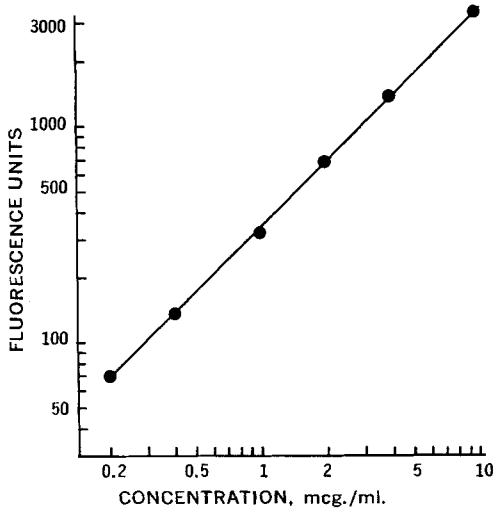


Figure 5—*Fluorometric standard curve obtained for ampicillin added to pooled human plasma.*

The penicilloic acid product of ampicillin is readily formed in the presence of the enzyme penicillinase. The ability of the fluorometric procedure to measure this product is demonstrated by the results of the study shown in Fig. 8. Microbiologically active ampicillin rapidly disappears in the presence of the enzyme, while fluorometrically the total apparent concentration remains constant. These data show that equimolar concentrations of both ampicillin and α-aminobenzylpenicilloic acid yield identical amounts of the fluorescent product. This indicates that either ampicillin degrades to the acid during the formation of the fluorescent product or that both compounds can be directly transformed into the same intermediate.

An attempt to separate the small quantity of degradation product from ampicillin in urine using chromatographic techniques was not successful. This was primarily due to the similar physicochemical properties of these compounds. Direct identification of the apparent metabolites of ampicillin in serum and urine was, therefore, not possible at this time.

DISCUSSION

The fluorometric assay for ampicillin described in this report is an indirect but highly sensitive method for the measurement of the antibiotic in solution. Although the precise chemical identity of the fluorescent product was not determined, various physicochemical properties of it, as well as of ampicillin, have been elucidated. Such information provides interesting insights into the chemistry of these penicillin derivatives.

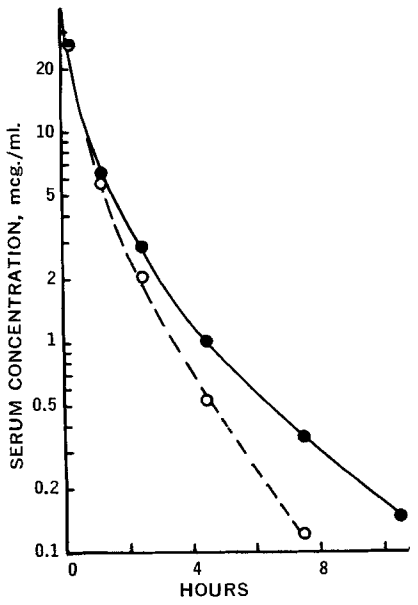


Figure 6—*Serum concentrations of apparent ampicillin as a function of time after intravenous administration of 570 mg. to a male subject. Concentrations were obtained with the fluorometric* (●) *and microbiological* (○) *methods.*

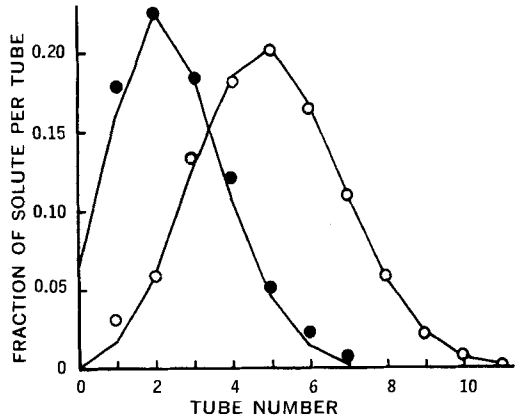


Figure 7—*CCD profile for the fluorescent product of ampicillin in urine using acetone-chloroform* (●) *and chloroform* (○) *solvents and pH 3.4 buffer. Lines represent theoretical calculations (9).*

Hou and Poole (11) were the first to note that aging of ampicillin mixtures includes the formation of a substance with absorption at 340–355 nm. This material was probably identical to the fluorescent product found in the present investigation. Based on UV absorption spectra, kinetic data, and other information, these investigators (11) as well as others (12) proposed various pathways of degradation of ampicillin. These mechanisms primarily involved cleavage of the β-lactam portion of the molecule, and the benzylamino group was assumed to remain intact. The results of the present study (Table II) have shown that both portions of the ampicillin molecule are required in the formation of the fluorescent product. Addition of formaldehyde, a type of formol titration (13), enhances the reaction by reducing the basicity of the amino group. A chemical pathway for formation of a fluorescent material which takes these factors into account is shown in Scheme II. The formation of a 3,6-substituted diketopiperazine (III) from either ampicillin (I) or α-aminobenzylpenicilloic acid (II) is consistent with the ability of amino acid esters to condense to form diketopiperazines (15). The imino protons of product III could subsequently dissociate in solution above pH 10 to produce a more fluorescent species, which would account for the pH–fluorescence profile of Fig. 2. It is possible for the remaining portion (R) of the piperazine molecule (III) to undergo a variety of other chemical reactions (11, 12) that are characteristic of penicillins rather than amino acids. Further physicochemical analysis of the fluorescent product will be required for direct confirmation of the suggested pathway.

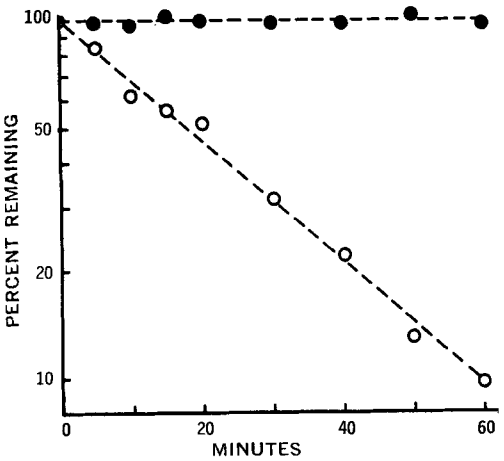
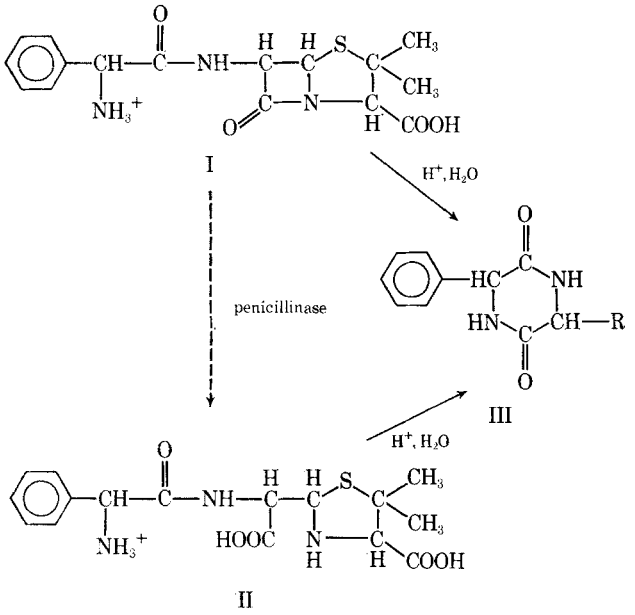


Figure 8—*Effect of penicillinase on ampicillin stability as a function of time as determined by the fluorometric* (●) *and microbiological* (○) *assays.*



Scheme II—Proposed pathway for formation of a 3,6-disubstituted diketopiperazine (III) from ampicillin (I) and/or α-aminobenzylpenicilloic acid (II)

The results of the experiment with penicillinase showed that α-aminobenzylpenicilloic acid and ampicillin can be detected equally well by the fluorometric assay. The major *in vivo* degradation products of other penicillins such as penicillin G and phenoxymethyl and phenoxyethyl penicillin have been shown to be their respective penicilloic acid derivatives (14). Since all penicillins share a labile β-lactam portion of the molecule, it is very likely that the detected degradation product of ampicillin in serum and urine of man is its penicilloic acid derivative.

A search of the literature did not reveal a direct method for analysis of ampicillin in biological fluids. The feasibility of a direct assay is limited by the poor solubility of the amphoteric compound in organic solvents. In addition, the isolation of small amounts of the unchanged antibiotic is hampered by its relative instability in solution. For these reasons, measurements of apparent ampicillin in serum and other biological samples are usually done by microbiological methods which are easily performed. However, such assays are often used with the assumptions that the microorganism is sensitive only to the unchanged drug and that metabolites of the antibiotic do not act as competitive inhibitors of the bacteriocidal properties of ampicillin. It was found in the present study that the procedure (5) utilizing *B. subtilis* is specific for ampicillin in the presence of its degradation products. The microbiological method is, therefore, useful in conjunction with the fluorometric method as a means of differentiating between ampicillin and its *in vivo* metabolites or degradation products. The detection and quantitation of the latter compounds are of importance since the allergenic properties of the penicillins have been ascribed to their degradation products (12).

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