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**Stabilization of Ampicillin Analogs in Aqueous Solution. I. Assay of
Ampicillin in Solutions containing Benzaldehyde by Iodine
Colorimetry and the Effect of Benzaldehyde
on the Stability of Ampicillin**

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The interaction of ampicillin and benzaldehyde in aqueous solution was investigated. A modified iodine colorimetry (I_2 -colorimetry) was used for the assay of ampicillin in solutions containing ampicillin and benzaldehyde. Thus, total ampicillin including bioactive ampicillin-like substances which are present in aqueous solution could be determined by I_2 -colorimetry after diluting the solution and adjusting it to pH 3.5–4.0. The time courses of degradation of ampicillin with benzaldehyde in phosphate buffer (pH 8.00) at 35°C were followed by this method, and the reaction was found to be pseudo-first-order. Further, it was found that benzaldehyde inhibited the degradation of ampicillin under these conditions.

In view of the above results and the changes in the ultraviolet spectra of the solution, it is concluded that ampicillin and benzaldehyde form a complex.

Keywords—degradation of ampicillin; stabilization of ampicillin with benzaldehyde; complex formation; I_2 -colorimetry; equilibrium state

Ampicillin is a well-known broad spectrum antibiotic, but its stability is affected by various materials. For example, the degradation of ampicillin in aqueous solution is accelerated by glucose,¹⁾ alcohols²⁾ and amines.³⁾ However, few detailed studies have been reported on the interactions between ampicillin and aldehydes. In this paper, the interaction of ampicillin with benzaldehyde was studied. We first aimed to modify the iodometry procedure used frequently as an assay method for penicillins. Secondly, we hoped to elucidate the effect of benzaldehyde on the stability of ampicillin in aqueous solution.

Experimental

Materials—Ampicillin sodium (Sigma Chemical Company), penicillin G potassium (Wako Pure Chemical Ind. Ltd.) were of guaranteed reagent grade. Benzaldehyde and all other chemicals were of the highest commercial grade available and were used without further purification.

Reagents— I_2 Solution: I_2 , 0.04 M in 3.2 M KI, was prepared according to the micro iodometric assay by Novick.

Dilute Iodine Solution (Dil. I_2 Solution): 0.04 M I_2 was diluted exactly 100 times and stored in the dark. Solutions of 1 N HCl and 1 N NaOH used were of analytical grade.

Buffer Solutions—0.05 M Phthalate (pH 4.00): 10.22 g of potassium hydrogen phthalate was dissolved in water to make 1000 ml.

0.1 M Phthalate: The pH of 0.1 M potassium hydrogen phthalate was adjusted to pH 4.00 by the addition of 1 N HCl at 35°C.

0.1 M Phosphate: 0.1 M NaH_2PO_4 and Na_2HPO_4 were mixed well at 35°C, to give a pH of 8.00. The ionic strength of each buffer was adjusted to 0.5 by the addition of KCl.

Apparatus—The pH values of solutions were measured using a Toa pH meter, model HM-18ET. The measurements of ultraviolet (UV) absorption spectra were carried out with Hitachi, model 340 machine.

Assay Method—From a solution containing 2.5×10^{-4} M ampicillin, two equal samples of 1 ml were pipetted into separate 25 ml graduated test tubes with glass stoppers and adjusted to pH 3.5–4.0 with HCl. The solutions were left to stand for 5 min, then 2 ml of 1 N NaOH was added to one of the tubes. After 20 min at room temperature, 2 ml of 1 N HCl, 5 ml of dil. I_2 solution and phthalate buffer (0.05 M) were added to make 25 ml. The test tube was stoppered and kept for 20 min in darkness at room temperature. The absorbance of the solution was measured at 350 nm.

To the other tube, 2 ml of 1 N NaOH and 1 N HCl, 5 ml of dil. I_2 solution and phthalate buffer (0.05 M) were added to make 25 ml. The solution was treated similarly and the absorbance was measured at 350 nm. The difference between the two absorbances (ΔA) represents the amount of iodine equivalent to the ampicillin present. When the concentration of ampicillin was higher than 2.5×10^{-4} M, this method caused decoloration resulting from the complete consumption of iodine by penicilloic acid. Then, the analysis was carried out by degradation with alkali followed by quantitative addition of dil. I_2 solution for color development of the solution.

Bioassay—Antibacterial activity was assayed by the cylinder plate method with *Staphylococcus aureus* 209P as a test organism and with crystalline ampicillin as the assay standard, using Brain Heart Infusion (Difco) agar.

Kinetic Procedures—Ampicillin, benzylpenicillin solution (2.5×10^{-4} — 2.5×10^{-3} M) buffered to pH 4.00 or 8.00 with and without benzaldehyde (2.5×10^{-3} — 2.5×10^{-2} M) were stored in a constant temperature bath which was regulated by a thermostat with $\pm 0.1^\circ\text{C}$ precision. At suitable time intervals, samples were withdrawn, cooled on ice and assayed immediately. The pH of the solution was determined at the experimental temperature initially and at the end of the experiment. No significant change in pH was observed.

Results and Discussion

Application of I_2 Colorimetry to the Determination of Ampicillin

Novick *et al.*⁴⁾ have presented a micro iodometric assay for the hydrolysis of penicillin by penicillinase. The concentration of penicillin was estimated by measuring the absorbance of the starch iodine complex at 620 nm. In a similar study, Cole⁵⁾ determined penicillin by using the absorbance of iodine at 228 nm. In the former case, the color development depended on conditions such as the type and concentration of starch, the technique of preparing starch solution and the pH of the solution. Further, the method did not give reproducible analytical data under given conditions. Thus, we improved the latter method to obtain a simple method by which penicillins could be determined directly from the absorbance of iodine used to titrate penicilloic acid. Since ampicillin and other chemicals did not decolorize iodine, the concentration of iodine could be calculated from the absorbance at 350 nm.

The absorbance of $ca. 10^{-5}$ M iodine at 350 nm was constant below pH 4.00 and no changes were observed during storage in darkness for 40 min. A linear relationship between the concentration of iodine below 5×10^{-5} M and the absorbance at 350 nm was observed at pH < 4.00. Various concentrations of ampicillin were treated by the procedure described in "Experimental," and the extent of iodine consumption (converted into absorbance, ΔA) was plotted against the concentration of ampicillin. A linear relationship was found as shown in Fig. 1.

Fig. 1. Typical Calibration Curve for Ampicillin

Fig. 2. Time Courses followed by I_2 -Colorimetry of the Degradation of Ampicillin with and without Benzaldehyde in 0.1 M Phosphate Buffer of pH 8.00 at 35°C and $\mu=0.5$

○, ampicillin 2.5×10^{-3} M; ●, ampicillin 2.5×10^{-3} M with benzaldehyde 2.5×10^{-2} M.

Similar results were obtained with benzylpenicillin. Further, benzaldehyde below $2.5 \times 10^{-2} \text{ M}$ did not consume iodine at all in this I_2 -colorimetry. Therefore, from the results shown in Table I also, it was clear that ampicillin and benzylpenicillin could be successfully determined by this I_2 -colorimetry.

I_2 -Colorimetry of the Degradation Process of Ampicillin with Benzaldehyde in Aqueous Solution

The time courses of the degradation of ampicillin was followed in phosphate buffer (pH 8.00) containing $2.5 \times 10^{-3} \text{ M}$ ampicillin with or without $2.5 \times 10^{-2} \text{ M}$ benzaldehyde at 35°C . The results in a solution containing only ampicillin followed pseudo-first-order kinetics as shown in Fig. 2.

In a solution with benzaldehyde, however, similar plots did not show a pseudo-first-order reaction and the initial quantitative values were lower than for ampicillin alone. In this run, as the concentration of ampicillin was higher than $2.5 \times 10^{-4} \text{ M}$, ampicillin was determined directly by I_2 -colorimetry (using I_2 solution diluted 10 times) without dilution. On the other hand, the time courses for benzylpenicillin of the same concentration in the buffers of pH 8.00 and 4.00 both followed pseudo-first-order kinetics, as shown in Fig. 3. In addition, no difference between the degradation rates with and without benzaldehyde was found at either pH.

Fig. 3. Pseudo First Order Plots for the Degradation of Benzylpenicillin with and without Benzaldehyde in Buffers of pH 4.00 and 8.00 at 35°C and $\mu = 0.5$

○, benzylpenicillin $2.5 \times 10^{-3} \text{ M}$; ●, benzylpenicillin $2.5 \times 10^{-3} \text{ M}$ with benzaldehyde $2.5 \times 10^{-2} \text{ M}$.

Fig. 4. Pseudo First Order Plots for the Degradation of Ampicillin with and without Benzaldehyde in 0.1M Phthalate Buffer of pH 4.00 at 35°C and $\mu = 0.5$

○, ampicillin $2.5 \times 10^{-3} \text{ M}$; ●, ampicillin $2.5 \times 10^{-3} \text{ M}$ with benzaldehyde $2.5 \times 10^{-2} \text{ M}$.

Figure 4 shows the time courses of the degradation of ampicillin in phthalate buffer (0.1 M) of pH 4.00. At this pH, the degradation of ampicillin followed apparent first-order kinetics regardless of the addition of benzaldehyde, and there was no difference in the degradation rates. That is, the application of I_2 -colorimetry for the study of the degradation of benzylpenicillin in aqueous solution did not show abnormal phenomena in the presence of benzaldehyde, while that of ampicillin in phosphate buffer of pH 8.00 showed abnormality upon the addition of benzaldehyde. Thus, there appears to be some interaction between ampicillin and benzaldehyde in the buffer of pH 8.00.

Figure 5 shows the time courses of the degradation of ampicillin in the buffer of pH 8.00 at 0°C with and without benzaldehyde. The concentrations of ampicillin without benzaldehyde were essentially constant from 98 to 101%, while those with benzaldehyde gradually decreased. However, antibacterial activity of ampicillin with benzaldehyde as determined by bioassay was little changed in the solution over 120 h as shown in Table I. Thus, these results indicate that ampicillin and benzaldehyde formed a bioactive complex in the buffer of pH 8.00 which could not be determined successfully by I_2 -colorimetry. Similar results were also obtained with hetacillin. A simultaneous determination of hetacillin and ampicillin

Fig. 5. Time Courses followed by I_2 -Colorimetry of the Degradation of Ampicillin with and without Benzaldehyde in 0.1 M Phosphate Buffer of pH 8.00 at 0°C and $\mu=0.5$

●, ampicillin 2.5×10^{-3} M; ○, ampicillin 2.5×10^{-3} M with benzaldehyde 2.5×10^{-2} M.

The points represent the median and vertical bars represent the standard deviations.

TABLE I. Determination of Ampicillin by I_2 -Colorimetry and Bioassay

	I_2 -colorimetry (mm)		Bioassay (mm)	
	24 h	120 h	24 h	120 h
Ampicillin alone	2.4	2.6	2.5	2.3
Ampicillin with benzaldehyde	2.2	2.0	2.6	2.8

Samples used for the analysis were 0.1 M phosphate buffer solutions of pH 8.00 containing 2.5×10^{-3} M ampicillin with and without 2.5×10^{-2} M benzaldehyde at 0°C.

was reported by the use of iodometry and by application of the resistance of hetacillin to alkaline hydrolysis.⁶⁾

The degradation time with alkali to penicilloic acid was extended in the buffer of pH 8.00 solution in which ampicillin had been allowed to react with benzaldehyde during 5 days at 0°C. The results (Table II) showed that the extent of iodine consumption increased gradually with time up to 80 min. Hence, total ampicillin containing the complex could not be determined by increasing the hydrolysis time.

On the other hand, the extent of iodine consumption increased with increase in alkali concentration upon analysis of the same solution. However, the data varied widely. Thus, it seemed that the abnormality observed in the degradation of ampicillin with benzaldehyde at pH 8.00, as shown in Fig. 2, occurred because the complex resulting from the reaction between ampicillin and benzaldehyde was not hydrolyzed to penicilloic acids which consume iodine quantitatively on I_2 -colorimetry.

Furthermore, since no abnormality of ampicillin at pH 4.00 or of benzylpenicillin at both pHs was observed, it is likely that the free α -amino group of ampicillin is involved in the formation of the complex.

TABLE II. I_2 Consumption in Terms of Absorbance for a Solution containing Ampicillin and Benzaldehyde followed by Direct I_2 -Colorimetry

Time of hydrolysis (min)	Absorbance, ΔA NaOH conc.		
	1 N	3 N	5 N
20	0.760	0.771	0.765
40	0.782	0.799	0.831
60 ^{a)}	0.811 ± 0.001	0.801 ± 0.022	0.874 ± 0.111
80 ^{a)}	0.843 ± 0.002	0.876 ± 0.018	0.922 ± 0.102

^{a)} The results are means of 10 measurements.

Samples used for the analysis were 0.1 M phosphate buffer solutions in which 2.5×10^{-3} M ampicillin had been allowed made to react with 2.5×10^{-2} M benzaldehyde at 0°C for 5 d.

After storage of the buffer solution of pH 8.00 containing 2.5×10^{-3} M ampicillin and benzaldehyde for 7 d at 0°C, the time courses of degradation in the solution diluted 10 times with the buffer of pH 8.00 or pH 4.00 were studied on storage at 0°C. A sample diluted with the buffer

of pH 4.00 gave, immediately after dilution, the same value as ampicillin itself. However, a sample diluted with the buffer of pH 8.00 gave a lower initial value than ampicillin itself but gave the same value as ampicillin after 2 h. It is thought that the complex dissociates into free ampicillin on dilution and that the dissociation is fast at pH 4.00. This suggests that the total ampicillin containing the complex can be determined by dilution. The results shown in Fig. 6 were obtained by examining the time courses in buffer of pH 8.00 in which 2.5×10^{-4} M ampicillin was allowed to react with 2.5×10^{-3} and 2.5×10^{-2} M benzaldehyde at 35°C. It was found that the formation of the complex increased with increase of benzaldehyde concentration and then the alkaline hydrolysis of the complex formed at 2.5×10^{-4} M ampicillin was insufficient for successful I_2 -colorimetry. Further, it was proved that such conditions as decreasing the concentration of ampicillin to 2.5×10^{-4} M, or adjusting the pH of the solution to 3.5–4.0, were required for determining total ampicillin including bioactive complex in a solution of ampicillin with benzaldehyde.

The time course of the degradation of ampicillin with 2.5×10^{-2} M benzaldehyde was followed by I_2 -colorimetry after adjusting the pH of the solution to 3.5–4.0. The plots of the logarithm of residual ampicillin *versus* time gave straight lines as shown in Fig. 6.

Degradation of Ampicillin with Benzaldehyde in Solution

The time courses of total ampicillin were examined by I_2 -colorimetry in buffer solution of pH 8.00 containing 2.5×10^{-4} M ampicillin and 2.5×10^{-3} , 1.0×10^{-2} and 2.0×10^{-2} M benza

Fig. 6. Time Courses followed by I_2 -Colorimetry of the Degradation of Ampicillin with and without Various Concentrations of Benzaldehyde in 0.1 M Phosphate Buffer of pH 8.00 and/or 0.1 M Phthalate Buffer of pH 4.00 at 35°C and $\mu=0.5$

○, ampicillin 2.5×10^{-4} M; △, ampicillin 2.5×10^{-4} M with benzaldehyde 2.5×10^{-3} M; ●, ▲, ampicillin 2.5×10^{-4} M with benzaldehyde 2.5×10^{-2} M.

Fig. 7. Pseudo-First-Order Plots for the Degradation of Ampicillin with and without Benzaldehyde in 0.1 M Phosphate Buffer of pH 8.00 at 35°C and $\mu=0.5$

○, ampicillin 2.5×10^{-4} M; ●, ampicillin 2.5×10^{-4} M with benzaldehyde 1.0×10^{-2} M; △, ampicillin 2.5×10^{-4} M with benzaldehyde 2.5×10^{-2} M.

TABLE III. Pseudo-First-Order Rate Constants for the Degradation of Benzylpenicillin and Ampicillin with and without Benzaldehyde at Various pH Values, 35°C and $\mu=0.5$

Penicillin	Benzaldehyde added	Rate constants ^{a)} (h ⁻¹)	
		pH 4.00	pH 8.00
Benzylpenicillin (2.5×10^{-4} M)	0	0.21	1.39×10^{-2}
	2.5×10^{-3} M	0.20	1.39×10^{-2}
Ampicillin (2.5×10^{-4} M)	0	9.15×10^{-3}	2.68×10^{-2}
	2.5×10^{-3} M	9.92×10^{-3}	2.41×10^{-2}
	1.0×10^{-2} M	—	1.97×10^{-2}
	2.5×10^{-2} M	—	1.59×10^{-2}

a) The rate constants were calculated from the analytical results obtained by I_2 -colorimetry.

dehyde at 35°C. As shown in Fig. 7, all the plots of the logarithm of residual ampicillin *versus* time showed a pseudo-first-order reaction and apparent stabilization compared to a solution of ampicillin alone. Further, increasing the concentration of benzaldehyde tended to increase the stabilization. Table III summarizes the pseudo-first-order rate constants under various conditions.

As can be seen from Table III, benzaldehyde inhibits the degradation of ampicillin by the formation of a complex in the buffer of pH 8.00. In addition, since the degradation of ampicillin with excess benzaldehyde in aqueous solution follows pseudo-first-order kinetics, it seems that the complex comes to equilibrium with ampicillin and the reversible equilibrium reaction rate is faster than the rate of degradation of ampicillin itself.

UV Spectra of Ampicillin with Benzaldehyde in Solution

To confirm that the complex which could be determined by I_2 -colorimetry was formed between ampicillin and benzaldehyde in the buffer of pH 8.00, UV spectra of the solution were examined. The buffer solution of pH 8.00 and solution of pH 4.00 (adjusted with HCl) containing $1.0 \times 10^{-2} M$ ampicillin and benzaldehyde were kept at 0°C and the changes of UV spectra of each solution were followed during aging (Fig. 8). UV spectra were measured immediately after diluting the solution 100 times with the buffers of each pH. As shown in Fig. 8, in the buffer of pH 8.00, the absorbance at 210 nm⁷⁾ increased, while the peak at 247 nm⁸⁾ shifted to 250 nm with decreasing absorbance, after 24 h. No changes of UV spectra were found in the subsequent measurements. Further, it seemed that the UV spectrum observed after 24 h was that of the complex.

On the other hand, the absence of changes of absorption spectra at pH 4.00 after 24 h indicated noncomplexation. Thus, these results were consistent with the kinetic data shown in Fig. 4. Furthermore, the initial absorption curve at each pH was in fair agreement with the sum of those of ampicillin and benzaldehyde. The buffer solution of pH 8.00 described

Fig. 8. Spectral Changes of a Solution containing $1.0 \times 10^{-2} M$ Ampicillin and Benzaldehyde at pH 8.00 or 4.00, at 0°C

—, at pH 8.00; ---, at pH 4.00.

Figures on the plot are time after mixing (in hour).

Fig. 9. UV Spectra of Ampicillin and Benzaldehyde at pH 4.00

—, ampicillin; ---, benzaldehyde.

The concentrations of ampicillin and benzaldehyde were $1.0 \times 10^{-2} M$ each.

above, after standing for 72 h at 0°C, was diluted 100 times with the buffer of pH 8.00 or hydrochloric acid solution of pH 4.00 and then the changes of UV spectra were measured.

As shown in Fig. 10, the absorbance at 210 nm decreased, while the peak at 250 nm shifted to 247 nm with increasing absorbance in a dilute solution of pH 8.00.

No changes of the absorption spectra, which possessed isosbestic points at 225 and 275 nm, were observed after 180 min. In hydrochloric acid solution of pH 4.00, similar changes of UV spectra were observed, and stopped after 3 min. Further, the latter absorption curve was consistent with sum of the individual ones, as shown in Fig. 8. These results indicate that the complex dissociates into ampicillin and benzaldehyde on dilution and that the dissociation rate at pH 4.00 is fast compared to that at pH 8.00.

Fig. 10. Spectral Changes of a Solution of 1.0×10^{-2} M Ampicillin with 1.0×10^{-2} M Benzaldehyde after Dilution with the Buffer of pH 8.00 or 4.00 at 0°C

Figures on the plot are time after mixing (in min).

Thus, it was demonstrated that total ampicillin containing the complex could be determined completely by adjusting the pH of the solution to 4.0 for I_2 -colorimetry.

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