[Chem. Pharm. Bull. 30(9)3310—3318(1982)]

Stabilization of Ampicillin Analogs in Aqueous Solution. III.[1-3] Kinetics of Degradation of Ampicillin with Furfural in Aqueous Solution and Physical Properties of Ampicillin-aldehyde Adducts

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(Received February 23, 1982)

The degradation of ampicillin was found to be inhibited due to the formation of a 1:1 molar adduct between ampicillin and furfural in alkaline solution. Further, specific base catalysis for the ampicillin-furfural adduct was smaller, about one hundredth, than that for ampicillin-benzaldehyde adduct.

Since the adduct formation was not observed at pH<6.00 and the formation constant of the adduct increased with increase of pH, the α-amino group of ampicillin was concluded to participate in the adduct formation. Thus, to estimate the structure of the adduct, various physical properties of samples prepared by freeze-drying of alkaline solution containing ampicillin and an aldehyde (furfural or benzaldehyde) were studied and compared. Nuclear magnetic resonance (NMR) spectra (^{1}H and ^{13}C), infrared (IR) spectra and the chemical properties of the adducts suggested that they have a Schiff base structure formed from the aldehyde moiety and the free amino group of ampicillin.

Keywords——ampicillin; furfural; ampicillin-furfural adduct; formation constant of adduct; Schiff base; freeze-dried product

In previous papers,[2,3] it was found that the degradation of ampicillin was inhibited by the adduct formation between ampicillin and benzaldehyde in alkaline solution.

In this paper, the interaction in aqueous solution between ampicillin and furfural (which is less toxic than benzaldehyde) was investigated, and the structure of the adducts formed between ampicillin and aldehyde was estimated from the physicochemical properties.

Experimental

Materials——Ampicillin sodium was described previously.[2] Furfural was of the highest commercial grade and was used after further purification by distillation under reduced pressure. All other chemicals were commercial products of the highest reagent grade and were used without further purification.

Reagents——All reagents used for I_2-colorimetry were described in the preceding paper.[2]

Buffer Solutions——The buffers used for degradation (pH 4—9) and determination (pH 4) were described in the preceding paper.[2] The pH of buffers was measured at the experimental temperature with a Toa pH meter, model HM-18ET.

Analytical Method——I_2-colorimetry[2] was used for ampicillin assay. Furfural in aqueous solution did not decolorize iodine. The values determined in solutions (pH 4.00 or 8.00) containing ampicillin and furfural by I_2-colorimetry were in good agreement with those obtained by bioassay and with those of the same concentration of ampicillin alone.

Kinetic Procedure——All kinetic investigations were carried out at 35 ± 0.1°C. Ampicillin was dissolved in a suitable buffer solution with or without furfural which had been preheated to the experimental temperature. The initial concentration of ampicillin was 2.5×10^{-4} M and that of furfural was $2.5\times10^{-3}-0.1$ M. At appropriate intervals, samples were withdrawn, cooled on ice and assayed for total intact ampicillin by I_2-colorimetry as described previously.

Preparation of Ampicillin-aldehyde Adduct——Aqueous solution (pH 8.0) containing 0.1 M ampicillin and 0.1 M aldehyde (furfural or benzaldehyde) was lyophilized after reacting for 72 h at 0°C.

Determination of Aldehyde in the Adduct prepared——The freeze-dried product (100 mg) was dissolved in water (10 ml). The pH was adjusted to 3—4 with 1 N HCl and the solution was extracted with three 6 ml portions of ether. The total extract was concentrated under reduced pressure, and the residue was dissolved in 0.1 M borate buffer (pH 8.00) to make 10 ml, then 2 ml of the solution was diluted exactly 10 times with the borate buffer.

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The resultant solution was analyzed by high performance liquid chromatography (HPLC), Waters HPLC system, model 6000/U6K (detection at 240 nm, flow rate of 1 ml/min, room temperature). μ-Bondapak C_{18} and 50% aqueous methanol were used as the stationary and mobile phases, respectively. Furfural and benzaldehyde were detected at 3.97 and 6.75 min, respectively. The amount of aldehyde was determined by comparing the peak area with that of a similary chromatographed standard.

Stability of Furfural in a Solution——Furfural (0.1 M) buffered to pH 4.00 or 9.00 was stored at 35°C and was determined periodically by measuring the absorbance at 278 nm with a Hitachi model 100-10 spectrophotometer.

Ultraviolet (UV) Spectral Measurements——UV absorption spectra of aqueous reaction mixtures were measured with a Shimadzu model 210A spectrophotometer.

Nuclear Magnetic Resonance (NMR) Measurements——The spectra were measured at 23°C on a 100 MHz NMR spectrometer (JEOL, model JNM FX-100). Samples were dissolved in dimethyl sulfoxide-d_6 at a concentration of *ca.* 6 w/v %. No interactions were observed between ampicillin or furfural and tetramethylsilane (TMS) in NMR spectroscopy, so TMS was used as an internal standard. The technique of addition of D_2O was also used.

Infrared (IR) Measurements——The spectra were obtained with a diffraction grating infrared spectrometer (JASCO model DS-701G) by the KBr method.

Bioassay——Antibacterial activity was assayed by the method and with the test organism described in the previous paper.[2]

Results and Discussion

Degradation of Ampicillin with Furfural in Aqueous Solution

The time courses for the degradation of intact ampicillin in 0.07 M phosphate buffer (pH 8.00, μ=0.5) containing ampicillin and various concentrations of furfural at 35°C are shown in Fig. 1. Each plot of the logarithm of residual percent *versus* time gave a straight line. Further, the slope decreased with increasing addition of furfural, suggesting that ampicillin tended to be stabilized by the additives.

The plots of the pseudo-first-order rate constants obtained from these against the concentration of furfural were similar to those for benzaldehyde, described in the previous paper.[3] Consequently, the change of the total concentration, $[A]_T$, of intact ampicillin in the solution is given by Eq. (1):

$$-\frac{d}{dt}[A]_T = k_a[A]+k_c[AF] \qquad (1)$$

where $[A]$=concentration of free ampicillin; $[AF]$=concentration of adduct; k_a=first-order rate constant of β-lactam cleavage of ampicillin; k_c=first-order rate constant of β-lactam cleavage of adduct.

If 1:1 adduct formation is assumed, the formation constant, K, can be expressed as follows:

$$K = \frac{[AF]}{[A][F]} \qquad (2)$$

where $[F]$ is the concentration of furfural.

From Eqs. (1) and (2), Eq. (3) is obtained:

$$-\frac{d}{dt}[A]_T = \left\{\frac{k_a}{1+K[F]}+\frac{k_c \cdot K[F]}{1+K[F]}\right\}[A]_T = k_{obs}[A]_T \qquad (3)$$

where k_{obs} is the observed pseudo-first-order rate constant of intact ampicillin.

Since furfural was hardly decomposed in 48 h at pH 4.00, or in 24 h at pH 9.00, at 35°C and was used in more than 40-fold excess over ampicillin, the concentration of furfural can be considered to be constant during the reaction.

The reversible reaction rates for 1:1 adduct formation seem to be very fast compared with the degradation rates of free ampicillin or adduct, because the degradation of ampicillin with

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furfural proceeds with apparent first-order kinetics as shown in Fig. 1. Thus, Eq. (3) applies, and k_{obs} is obtained as follows.

$$k_{obs}=\frac{k_a}{1+K[F]}+\frac{k_c\cdot K[F]}{1+K[F]} \tag{4}$$

Eq. (4) is converted to Eq. (5):

$$\frac{k_a}{k_a-k_{obs}}=\frac{1}{qK}\cdot\frac{1}{[F]}+\frac{1}{q} \tag{5}$$

where $q=1-k_c/k_a$.

The double reciprocal plot[4] based on Eq. (5) is shown in Fig. 2.

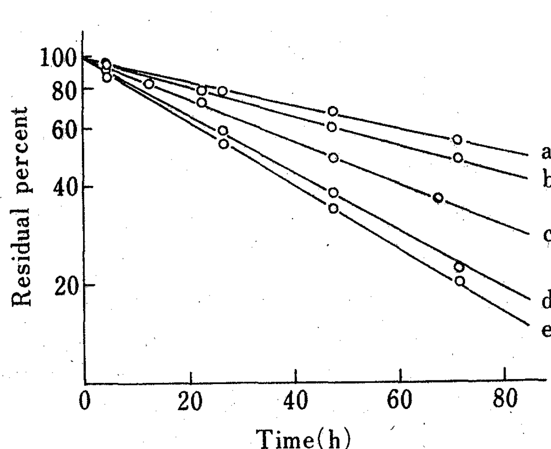

Fig. 1. First-order Plots for the Degradation of Ampicillin in the Presence and Absence of Furfural in 0.07 M Phosphate Buffer (pH 8.00) at 35°C and $\mu=0.5$

Ampicillin conc.: 2.5×10^{-4} M; furfural conc.: a=0.10, b=5.0×10^{-2}, c=2.5×10^{-2}, d=1.0×10^{-2} and e=0 M.

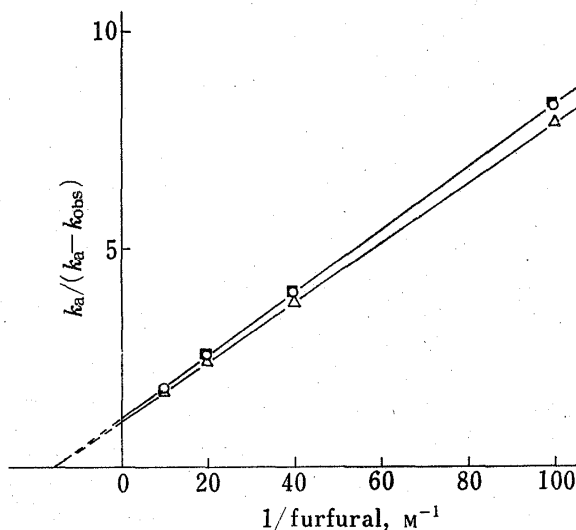

Fig. 2. Double Reciprocal Plots for Adduct Formation in Various Phosphate Buffer Concentrations (pH 8.00)

○, 0.11 M; ■, 0.07 M; △, 0.03 M.

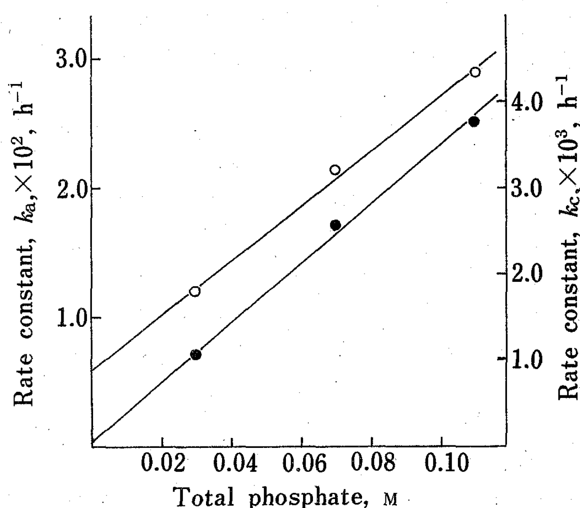

Fig. 3. Plots of Rate Constant, k_a or k_c, *versus* Buffer Concentration at pH 8.00

○, k_a; ●, k_c.

In each case, since the relation between $k_a/(k_a-k_{obs})$ and $1/[F]$ was linear, $1/q$ and $1/qK$, 1.14 and 0.072, were calculated by the least-squares method from the slope and intercept, respectively. The resulting values of k_c and K were $2.65\times10^{-3}\ h^{-1}$ and $15.8\ M^{-1}$, respectively. The results obtained in 0.03 and 0.11 M phosphate buffer (pH 8.00) are also shown in Fig. 2. Each double reciprocal plot was linear and the k_c and K values at each buffer concentration are summarized in Table I.

As shown in Table I, the formation constant, K, was constant ($16.1\ M^{-1}$) irrespective of the concentration of buffer, whereas k_c became larger with increase of the buffer concentration. Plots of k_c and k_a *versus* buffer concentration were linear,

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Table I. Formation Constant and Various Rate Constants of Ampicillin (k_a, k_a^0, k_{ap}) and Its Furfural Adduct (k_c, k_c^0, k_{cp}) in Phosphate Buffer at pH 8.00, 35°C, and μ=0.5

Buffer conc. M	10^{-2} rate constants						K M^{-1}
	k_a h^{-1}	k_a^0 [a] h^{-1}	k_{ap} [a] $M^{-1}h^{-1}$	k_c h^{-1}	k_c^0 h^{-1}	k_{cp} $M^{-1}h^{-1}$	
0		0.60			0.0035		
0.03	1.18		21.0	0.097		3.45	16.0
0.07	2.16			0.265			15.8
0.11	2.86			0.373			16.5

a) The values were in good agreement with those described in ref. 14. k_{ap} and k_a^0 are the phosphate buffer catalytic rate constant and the buffer-free rate constant of ampicillin, respectively.

as shown in Fig. 3.

This indicates that not only ampicillin but also its adduct with furfural is hydrolyzed by general acid-base catalysis, as is the case with the benzaldehyde adduct.[3] Thus, Eq. (6) holds.

$$k_c = k_{cp}[P] + k_c^0 \qquad (6)$$

where $[P]$ is total phosphate buffer concentration, and k_{cp} and k_c^0 are the catalytic rate constant and the buffer-free rate constant of the adduct, respectively. From the results in Fig. 3, k_{cp} and k_c^0 were $3.45\times10^{-2}\,M^{-1}\,h^{-1}$ and $0.035\times10^{-3}\,h^{-1}$, respectively. Thus, it was proved that the smaller catalytic effect of phosphate on the adduct (k_{cp}) and the slower degradation rate of the adduct (k_c^0) contributed to the stabilization of ampicillin by the addition of furfural in pH 8.00 buffer solution.[3]

Effect of pH on Kinetic Parameters of Ampicillin-furfural Adduct in Aqueous Solution

Ampicillin-furfural adduct was not formed by the addition of furfural in the region, pH $<$6.00 (Fig. 4). However, the pseudo-first-order rate constant of ampicillin degradation with furfural became smaller with increase of furfural concentration at above pH 7.00.

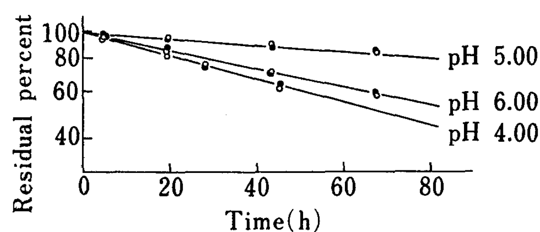

Fig. 4. First-order Plots for the Degradation of Ampicillin in the Presence (●) and Absence (○) of Furfural at Various pH Values, 35°C, and μ=0.5

The buffers used were 0.1 M phthalate (pH 4.00), 0.1 M acetate (pH 5.00) and 0.1 M phosphate (pH 6.00). Initial concentrations of ampicillin and furfural were 2.5×10^{-4} and 2.5×10^{-2} M, respectively.

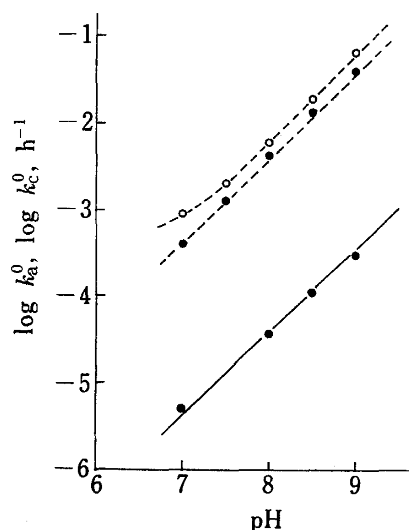

Fig. 5. Log k–pH Profile for the Degradation of Ampicillin (○) and Adduct (●) at 35°C and μ=0.5

The solid line was calculated from Eq. (9) using $k_{c\cdot OH}$, 16.60 $M^{-1}\,h^{-1}$ and the dashed lines are the log k_a–pH for ampicillin and log k_c–pH for benzaldehyde adduct, taken from ref. 3. The points are experimental values.

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The kinetic parameters were obtained from the double reciprocal plot at pH 7.00 by using Eq. (6) and the methods described previously.

Borate buffer was used for the studies at pH 8.50 and 9.00. No catalytic effects of buffer were observed in terms of the apparent degradation rates of ampicillin, regardless of the addition of furfural as mentioned in the previous paper,[3] while the rate constants became smaller with an increase of furfural for the same reasons as at pH 8.00.

Plots of the logarithm of the buffer-free degradation rate constant, k_c^0, of the adduct (Table II) *versus* pH resulted in a straight line with a slope of unity as shown in Fig. 5.

Table II. Formation Constant and Buffer-free Rate Constants of Ampicillin and Its Furfural and Benzaldehyde Adducts at Various pH Values at 35°C and $\mu=0.5$

Constant	pH						
	4	5	6	7	8	8.5	9
$k_a^0 \times 10^2\ h^{-1}$				0.10	0.60	1.75	5.37
$k_c^{0a)} \times 10^4\ h^{-1}$	0	0	0	0.055	0.35	1.10	2.50
$k_c^{0b)} \times 10^2\ h^{-1}$	0	0	0	0.038	0.44	1.38	4.17
$K^{a)}\ M^{-1}$	0	0	0	8.14	16.1	17.3	17.6
$K^{b)}\ M^{-1}$	0	0	0	48	76.9	85	86.8

a) These values were obtained from this run.
b) These values are those for the benzaldehyde adduct, taken from the previous paper.[3]

Thus, k_c^0 can be expressed as follows:

$$k_c^0 = k_{c\cdot OH} \cdot a\text{OH} \quad (7)$$

where $k_{c\cdot OH}$ and aOH are specific rate constant for the degradation of adduct and the activity of hydroxy ion, respectively.

Eq. (8) is obtained from Eq. (7).

$$\log k_c^0 = \log k_{c\cdot OH} - pK_w + \text{pH} \quad (8)$$

Here, as pK_w is 13.68[5] at 35°C, a value of 16.60 $M^{-1}\ h^{-1}$ was obtained for $k_{c\cdot OH}$ by substituting the results of Fig. 5 into Eq. (8). As has been described in the preceding paper,[3] $k_{c\cdot OH}$ for the benzaldehyde adduct was 1995.3 $M^{-1}\ h^{-1}$ and was about 100 times that for the furfural adduct. It seems that nucleophilic attack of hydroxy ion on the β-lactam carbonyl carbon atom of furfural adduct became less favorable than in the benzaldehyde adduct because of the enhancement of amino resonance and/or the larger steric hindrance in the furfural adduct.[6] Further, the degradation of the adduct was different from that of ampicillin at pH 7.00 as can be seen in Fig. 5. This supports the assumption that hydroxy ion catalysis for the adduct is also effective at pH 7.00.

Relationship between Formation Constant and pH

As is shown in Table II, the apparent formation constant, K, increased with increase of pH. Because adduct formation was not observed in the acidic region, an adduct was assumed to form between furfural and the anionic species, $[A^-]$, of ampicillin. Thus, the absolute formation constant, K_s, can be written as follows:

$$K_s = \frac{[AF]}{[A^-][F]} \quad (9)$$

The dissociation constant, K_a (8.91×10^{-8} M),[3] of ampicillin is as follows:

$$K_a = \frac{a\text{H}[A^-]}{[AH^\pm]}$$

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Thus, Eq. (10) can be derived from Eqs. (2) and (9)[3]:

$$K = K_s \frac{K_a}{K_a + aH} \qquad (10)$$

A value of K_s of 17.8 M^{-1} was obtained from the K value at each pH listed in Table II by means of a reciprocal plot based on Eq. (10).

The calculated and observed values shown in Fig. 6 (solid line and points, respectively) were in good agreement with each other.

This phenomenon were similar to that[3] of the benzaldehyde adduct, but the absolute formation constant, K_s, of the furfural adduct was about one-tenth of that of the benzaldehyde adduct.

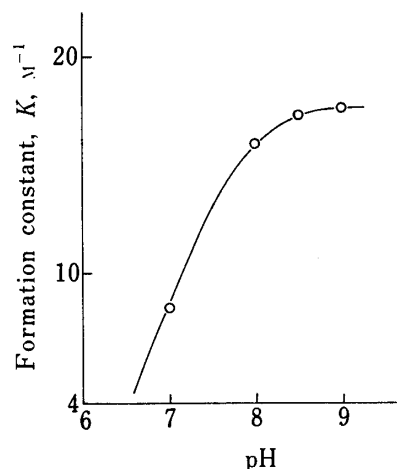

Fig. 6. Plot of the Formation Constant, K, of Ampicillin-furfural Adduct as a Function of pH in Aqueous Solution at 35°C and $\mu = 0.5$

The solid line was calculated from Eq. (11) using K_s, K_a and aH by the least-squares method, while the points represent experimental values.

UV Absorption Spectra of Adduct

The buffer solution of pH 8.00 and solution of pH 4.00 (adjusted with hydrochloric acid) containing 1.0×10^{-2} M ampicillin and furfural were kept at 0°C and the changes of UV absorption spectra were followed (Fig. 7).

The measurement was carried out immediately after diluting the solution 100 times with buffer. As can be seen in Fig. 7, the peaks at λ_{max} 209 and 277 nm shifted to 215 and 280 nm, respectively, after 24 h at pH 8.00. This change seems to reflect the adduct formation.

On the other hand, the absence of changes of absorption spectra at pH 4.00 after 24 h indicated that no adduct formation[2] had occurred. This absorption spectra at pH 4.00 was consistent with the sum of those of ampicillin and furfural (Fig. 8). Furthermore, no changes of biological activities in the reaction solution were recognized regardless of the presence of furfural during aging.

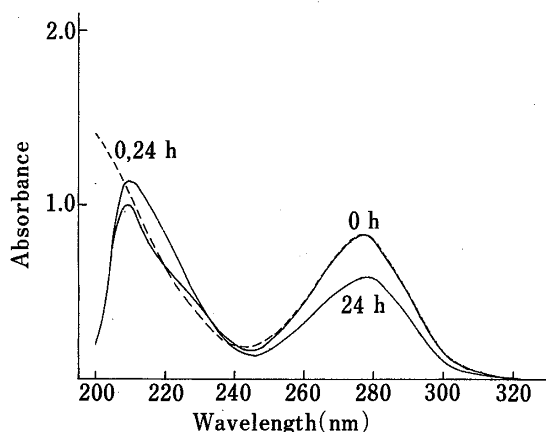

Fig. 7. Spectral Changes of a Solution containing 1.0×10^{-2} M Ampicillin and Furfural at pH 8.00 or 4.00, at 0°C

——, at pH 8.00; ----, at pH 4.00.
Figures on the plot are time after mixing (in h).

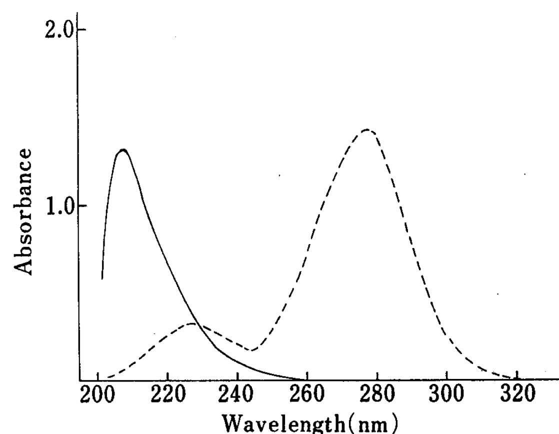

Fig. 8. UV Spectra of Ampicillin and Furfural at pH 4.00

——, ampicillin; ----, furfural.
The concentrations of ampicillin and furfural were 1.0×10^{-4} M each.

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Amorphous powder[7] which was obtained by freeze-drying of the solution (pH 8.0) containing ampicillin and furfural was used for the spectral run. The changes of absorption spectra of a solution containing 1.0×10^{-2} M freeze-dried sample were followed. UV absorption spectra were measured immediately after diluting the solution 100 times with buffer of pH 4.00 or 8.00.

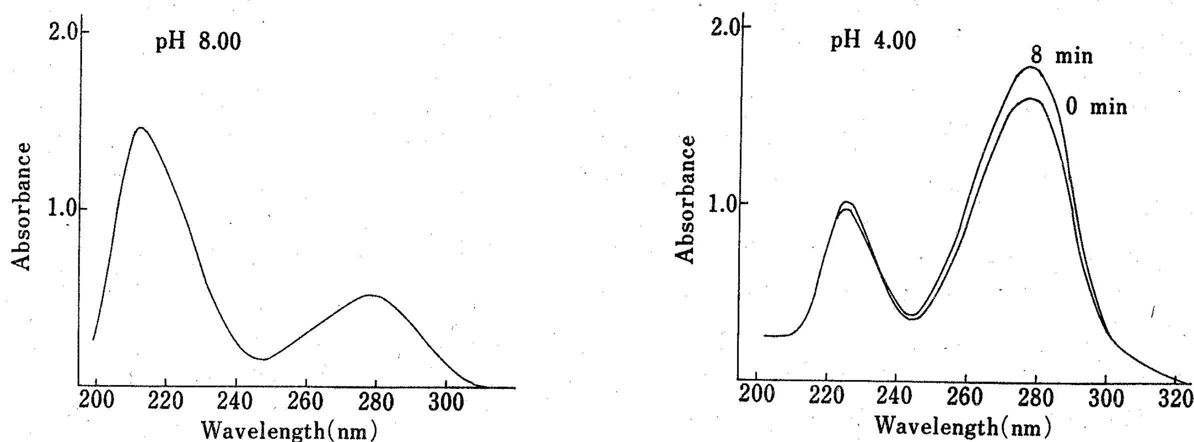

Fig. 9. Spectral Changes of a Solution of 1.0×10^{-2} M Freeze-dried Product after Dilution with Buffer of pH 8.00 or 4.00 at 0°C

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

As shown in Fig. 9, in the buffer of pH 4.00, the peak at 215 nm shifted to 210 nm with a decrease of absorbance, while the peak at 280 nm shifted to 277 nm with an increase of absorbance; there was an isosbestic point at 235 nm. No changes of these absorption spectra were observed after 8 min.

On the other hand, in a dilute solution of pH 8.00, the absorption spectrum immediately after dilution was very similar to that of the preceding solution which was allowed to react at 0°C for 24 h, and this spectrum changed to that of the initial buffer solution of pH 8.00 at 0°C (Fig. 7) after about 6 h. It was found from the above results that the freeze-dried products were amorphous materials formed in alkaline solution of ampicillin and furfural, and that the products dissociated completely into ampicillin and furfural under acidic conditions (pH 4.0).

Estimation of the Chemical Structure of the Adduct

The HPLC method described in "Experimental," was used to analyze the furfural and benzaldehyde contents of the freeze-dried products which were prepared from the solutions containing ampicillin and aldehyde. Furfural, 1.06×10^{-4} and benzaldehyde, 5.1×10^{-5} mol were obtained from 2.20×10^{-4} mol of each product. The products seemed to be mixtures of ampicillin and adduct, since no differences were observed in biological activities between the freeze-dried products and ampicillin, and further, these aldehydes were not contained in free form in the products.

Attempts to separate and purify these two compounds gave unsatisfactory results. Thus, to estimate the structure of the adducts, the freeze-dried products were dissolved in deuterated dimethyl sulfoxide with or without deuterium oxide, and their ^{1}H and ^{13}C NMR spectra were measured.

The ^{1}H NMR spectrum of ampicillin showed a broad signal at δ 8.63, assignable to amino protons (–NH and $-NH_2$), which disappeared on the addition of deuterium oxide.

In the case of the freeze-dried products, however, a newly observed proton signal at δ 8.17 (1H, in the furfural adduct) or at δ 8.40 (1H, in the benzaldehyde adduct) disappeared in the

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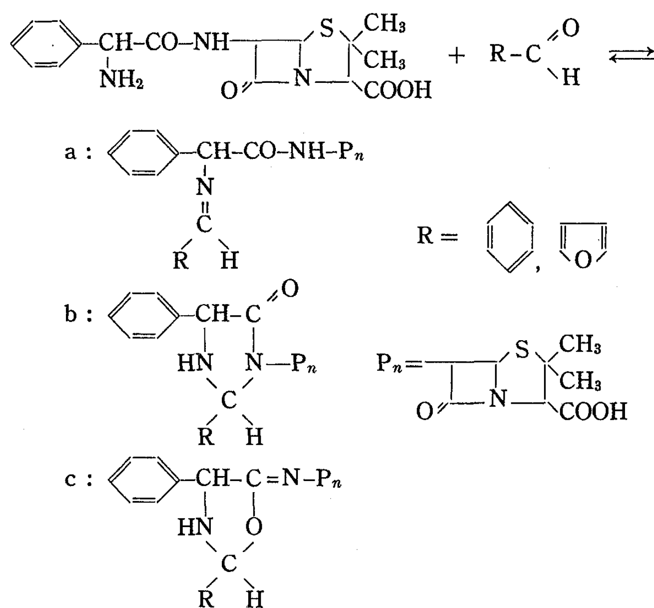

Chart 1

presence of deuterium oxide, and no changes of other signals were observed. This suggests the involvement of the α-amino group of ampicillin in adduct formation.

Further, the aldehyde proton at δ 9.66 of furfural or at δ 10.06 of benzaldehyde was not detected the corresponding adduct at δ 9—11. This indicates involvement of the aldehyde group in adduct formation. The furfural adduct showed three protons at δ 7.04—8.28 (furan ring).

The ^{13}C NMR spectrum showed carbonyl carbons at δ 170.3, 173.4, 173.7 (s) for ampicillin, and at δ 170.1 (2C), 173.0 (s) for the furfural adduct. Further, ampicillin showed four signals at δ 57.1, 58.3, 66.8, 73.9 (d) due to aliphatic tertiary carbons, whereas five signals were observed in the furfural adduct at δ 57.5, 61.1, 66.9, 73.6, 74.7 (d). It appears that the number of carbonyl carbons is the same in these compounds, while there is one more aliphatic tertiary carbon in the adduct. The signals due to these carbonyl and aliphatic tertiary carbons were also observed at similar chemical shift regions in the benzaldehyde adduct, but could not be assigned clearly because of overlapping with those of ampicillin.

In contrast, the aldehyde carbon of furfural (δ 178.6 (d)) or benzaldehyde (δ 193.2 (d)) was not observed in the corresponding adduct in this region of chemical shift (δ 180—200). These results, which are in good agreement with the ^{1}H NMR data, suggest that furfural or benzaldehyde is not involved in a free form in the adduct.

Since the disappearance of the ^{1}H signal upon addition of deuterium oxide seemed to contraindicate an enamine structure,[8] three other structures (a, b, and c in Chart 1) were considered for the adduct.[9]

The IR spectra of the freeze-dried prod-

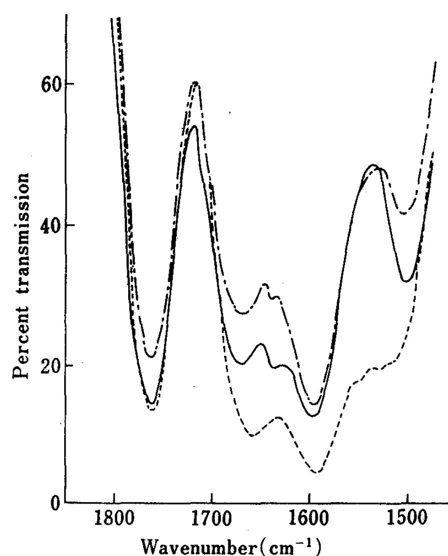

Fig. 10. IR Spectra of Freeze-dried Products

----, ampicillin; ——, furfural adduct; -·-·-, benzaldehyde adduct.

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ucts showed bands due to lactam at 1770 cm^{-1}, amide at 1680 cm^{-1} and carboxylate at 1600 cm^{-1}, and a characteristic stretching vibration due to azomethine at 1640 cm^{-1} [10] (Fig. 10). This azomethine band was not observed in freeze-dried ampicillin. Thus, the imidazolidinyl structure (b) was rejected. A band due to the furan ring at 880 cm^{-1} was detected in the furfural adduct, and no band due to aromatic aldehyde (1700 cm^{-1}) could be detected in either adducts, as shown in Fig. 10.

As discussed above, the adduct was not formed in acidic aqueous solution and the freeze-dried products dissociated into the original compounds within a short time under acidic conditions. The stability properties of hetacillin, which has the imidazolidinyl structure (b), are quite different from those of the adduct, that is, hetacillin is very stable at $pH<3.0$ compared to ampicillin.[11]

Davis and Levy[12] reported that an unsubstituted analog, the oxazolidine structure (c), easily hydrolyzed with cold water back to the amide. The adduct which was formed between ampicillin and aldehyde did not show such reactivity.

On the other hand, it is well known that the Schiff base is easily hydrolyzed under acidic conditions.[13]

Consequently, the ampicillin-aldehyde adducts may be the Schiff bases (a) shown in Chart 1.

Acknowledgement The authors thank Mr. M. Teranishi for NMR spectral measurements.

References and Notes

1) Presented in part at the 101st annual meeting of the Pharmaceutical Society of Japan, Kumamoto, April, 1981. Since the structure of the complex was considered to be a Schiff base, expressions such as adduct, adduct formation, formation constant were used in this paper in place of the terms complex, complexation, and stability constant used previously.
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