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Formation and Hydrolysis of Enamine in Aqueous Solution

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A mixture of sodium phenylalanine and ethyl acetoacetate in aqueous solution underwent enamine formation as determined by nuclear magnetic resonance- and ultraviolet-spectroscopic measurements. An equilibrium state was observed in the aqueous solution between the enamine and the starting materials. Although the observed equilibrium constant, K_{obs}, showed dependency on the pH value of the solution, the equilibrium constant, K, was not influenced by the pH value. The effect of pH values on K_{obs} was related to the pK_a value of phenylalanine, suggesting that only the unionized form of the amino moiety of phenylalanine could take part in the enamine formation.

Keywords——phenylalanine enamine; enamine formation; enamine ^{13}C-NMR; enamine UV absorbance; equilibrium constant

Since it has been reported that phenylalanine and phenylglycine enamines of ethyl acetoacetate enhance the rectal absorption of insulin,[1-3] many studies have been done on the adjuvant action of enamines[4-6] for the rectal absorption of poorly absorbable drugs and on pharmaceutical approaches for the preparation of rectal delivery dosage forms containing enamines as absorption promoters.

Because it is well known that, in general, enamines are unstable in aqueous solution,[7] it is necessary to clarify the behavior of enamines in aqueous solution and in biological fluid to prepare suitable dosage forms and to optimize the enhancing efficacy for rectal drug absorption. We have already reported[6] that the administration of an aqueous solution of cefmetazole with sodium phenylalanine and ethyl acetoacetate resulted in a remarkable rectal absorption of cefmetazole, while insignificant enhancing action was observed by the coadministration of cefmetazole with either phenylalanine or ethyl acetoacetate alone, suggesting a possible enamine formation in the aqueous solution under alkaline conditions.

In the present report, the formation and stability of enamine in aqueous solution were studied at various pH's using sodium DL-phenylalanine and ethyl acetoacetate. Factors influencing the formation of the enamine in aqueous solution were also studied.

Experimental

Materials——Sodium DL-phenylalanine was routinely prepared by a neutralization method from DL-phenylalanine (Wako Pure Chemicals Co., Ltd., Osaka, Japan) and ethyl acetoacetate was purchased from Wako Pure Chemicals Co., Ltd. Sodium salt of the phenylalanine enamine of ethyl acetoacetate was prepared by the method reported in previous paper.[4,6] Other reagents used were of analytical grade.

Ultraviolet (UV) Spectrophotometric Study——Studies of the formation and hydrolysis of the enamine in aqueous solution were carried out at various pH's with 0.1 M borate buffers (pH 7.5 to 9.75) or 0.1 M phosphate buffer (pH 7.0) at 37 °C. The concentration of enamine was determined at 288 nm with a UV spectrophotometer (Shimadzu UV-200) after more than 100-fold dilution with methanol. In a preliminary experiment, it was found that enamine

was fairly stable in hydrated methanol containing less than 5% water and enamine hydrolysis and formation were not observed in the methanol solution at 10 °C for the experimental period of spectrophotometric measurements.

^{1}H-Nuclear Magnetic Resonance (NMR) (90 MHz) and ^{13}C-NMR (22.5 MHz) Studies——NMR spectra were measured with a JEOL FX-90Q spectrometer with sodium 3-trimethylsilylpropionate, [2,2,3,3-D_4], as an internal standard.

Results

The maximum absorbance of phenylalanine enamine of ethyl acetoacetate in methanol was at 288 nm, with a large molar absorbance (ε) of $4\times10^4\,\text{M}^{-1}\,\text{cm}^{-1}$ (Fig. 1). Since the absorbance of sodium phenylalanine ($\varepsilon=1.33\,\text{M}^{-1}\,\text{cm}^{-1}$) and ethyl acetoacetate ($\varepsilon=18\,\text{M}^{-1}\,\text{cm}^{-1}$) at 288 nm in methanol can be ignored in comparison to that of the enamine, hydrolysis and formation of the enamine fraction in aqueous solution was determined by measuring the decrease or increase in absorbance at 288 nm as described in the experimental section.

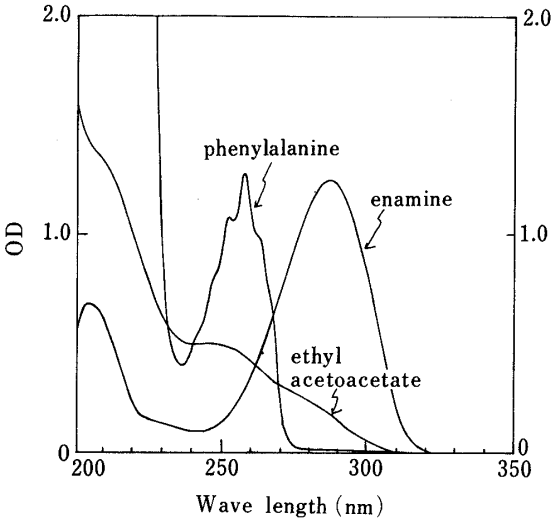


Fig. 1. UV Spectra of Sodium Phenylalanine Enamine of Ethyl Acetoacetate (5×10^{-5} M), Sodium Phenylalanine (6.7×10^{-3} M), and Ethyl Acetoacetate (1×10^{-2} M) in Methanol Containing 0.5% Distilled Water

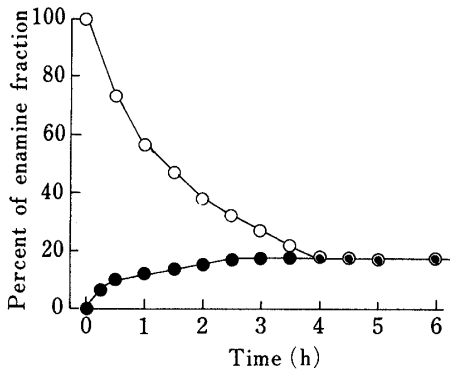


Fig. 2. Hydrolysis (○) and Formation (●) Profiles of Enamine in Aqueous Solution as a Function of Time of Incubation at pH 9.0 and at 37 °C

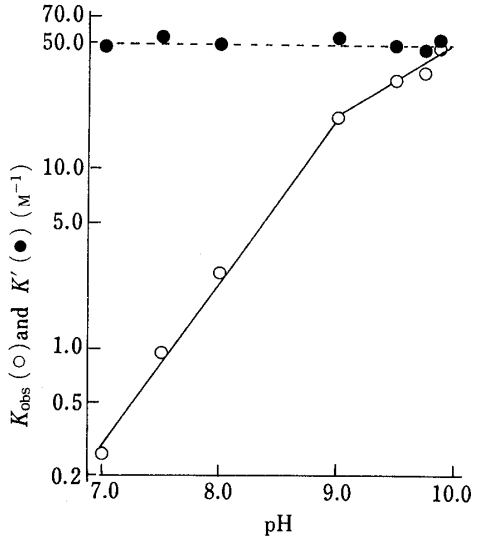


Fig. 3. Observed Equilibrium Constant (K_{obs}, ○) and Equilibrium Constant ($K'=K/[H_2O]$, ●) of Enamine Formation of Phenylalanine with Ethyl Acetoacetate at Various pH Values and at 37 °C

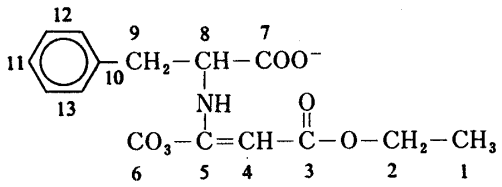
K_{obs} and K' are shown on log scales. Initial concentration of both phenylalanine and ethyl acetoacetate was 0.01 M.

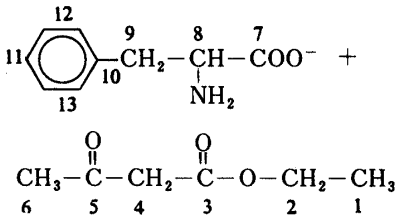
To study the hydrolysis of the enamine in aqueous solution, a 0.01 M aqueous solution of sodium salt of the phenylalanine enamine of ethylacetoacetate was incubated at 37 °C and the absorbance at 288 nm was measured (Fig. 2). The enamine fraction decreased following apparent first-order kinetics and the decrease terminated after 4 h, suggesting the existence of a possible equilibrium between hydrolysis and formation of the enamine in aqueous solution. This equilibrium state was sustained during the experimental period. To confirm the existence of the equilibrium of enamine, an aqueous solution of 0.01 M sodium phenylalanine and 0.01 M ethyl acetoacetate was incubated at 37 °C and the absorbance at 288 nm was followed. The absorbance gradually increased and reached a plateau after 4 h (Fig. 2). The fraction of enamine formed at the equilibrium state was the same as that found in the hydrolysis study when the same concentrations were employed.

From the values of enamine fraction found in the aqueous solution at the equilibrium state, an observed equilibrium constant (K_{obs}) can be obtained by assuming that the constituents present in the solution are phenylalanine, ethyl acetoacetate and the enamine. The observed equilibrium constant of enamine showed a pH-dependency (Fig. 3). In the pH range of 7.0 to 9.75, the lower the pH, the smaller the K_{obs} (the final pH of each solution 6 h after the start of incubation was not changed from the initial pH).

When a 1.0 M aqueous solution of enamine was used, the enamine fraction at pH 9.5 and 37 °C was estimated to be more than 85% at the equilibrium state by a simple calculation employing K_{obs}. Thus, to assign the NMR signals of the enamine in aqueous solution at pH

TABLE I. ^{13}C-NMR Spectra of the Phenylalanine Enamine of Ethyl Acetoacetate, Phenylalanine, and Ethyl Acetoacetate at pH 9.5 in D_2O at 37 °C





Number of carbon	Chemical shift, ppm (in D_2O)		
	Enamine	Phenylalanine	Ethyl acetoacetate
1	16.5 (q)[a]		16.5 (q)
2	61.6 (t)		65.6 (t)
3	173.5 (s)		172.7 (s)
4	82.6[b] (d)		52.8 (t)
5	165.3 (s)		205.9 (s)
6	21.2 (q)		33.1 (q)
7	181.1 (s)	184.1 (s)	
8	62.7 (d)	60.0 (d)	
9	42.9 (t)	43.3 (t)	
10	140.3 (s)	140.9 (s)	
11	129.0 (d)	129.2 (d)	
12	131.9 (d)	131.9 (d)	
13	130.9 (d)	131.2 (d)	

a) Abbreviations given in parentheses denote the signal patterns observed in off-resonance experiments: d = doublet, q = quartet, s = singlet, t = triplet.

b) This signal was not observed due to D exchange. The assignment was made from the spectrum in H_2O (with d_6-dimethyl sulfoxide as the lock signal).

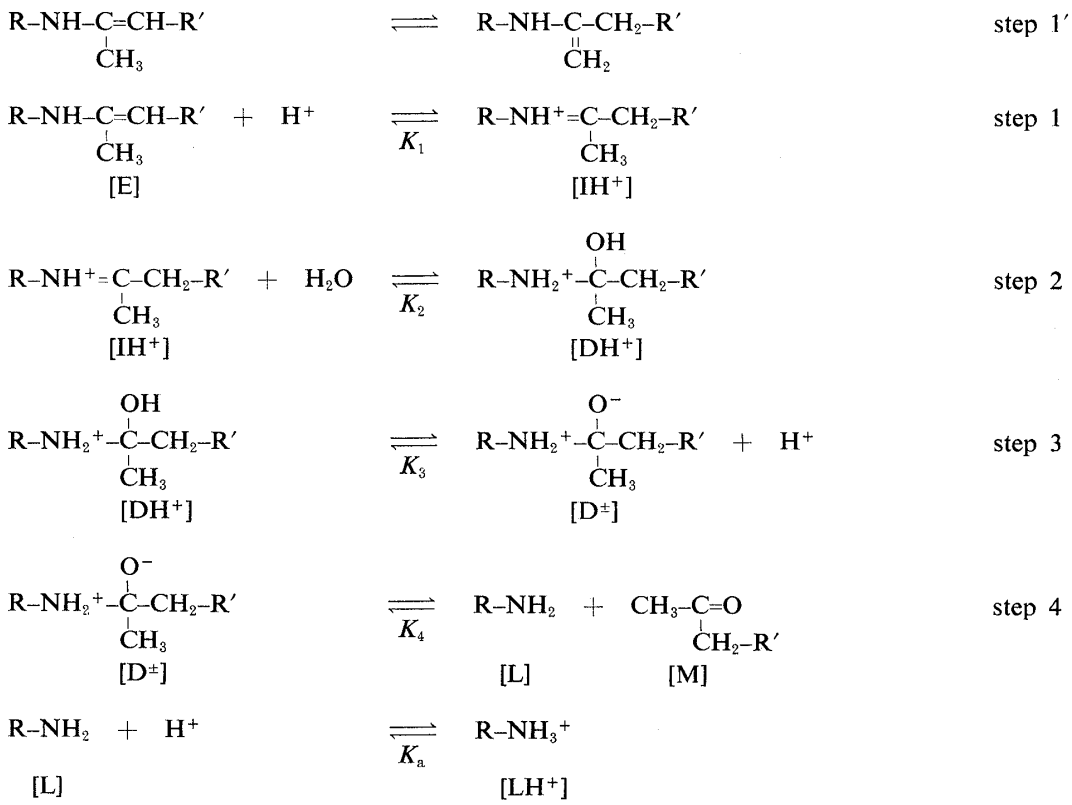
9.5, a 1.0 M aqueous solution of enamine was employed. The NMR study of the formation of enamine was carried out using 0.3 M each of phenylalanine and ethyl acetoacetate at pH 9.5 (the enamine fraction under these conditions was estimated to be about 75% in the equilibrium state), because the solubility of ethyl acetoacetate in aqueous solution at pH 9.5 and 37 °C was less than 0.5 M.

The signals in the ^{13}C-NMR spectra of the enamine, phenylalanine and ethyl acetoacetate were assigned as shown in Table I. The signals of C-4 (82.6 ppm), C-5 (165.3 ppm) and C-6 (21.1 ppm) of the enamine were characteristic, so that the presence of the enamine in a mixture of phenylalanine and ethylacetoacetate in aqueous solution could be easily detected.

In the ^{1}H-NMR spectrum of the enamine taken in D_2O at 37 °C, the intensity of the signal due to 6-CH_3 (1.64, 3H, s) gradually decreased and the protons at C-4 and C-6 were presumed to be exchanged with D under these conditions. This was confirmed by the following findings. In the ^{1}H-NMR spectrum (in $CDCl_3$) of ethyl acetoacetate, which was obtained by ether extraction of the above D_2O solution of enamine, weak signals due to the protons at C-4 and C-6 of ethyl acetoacetate were also observed, suggesting a partial exchange with D.

Next, in order to avoid complications due to the D_2O exchange, the ^{1}H- and ^{13}C-NMR analyses were carried out in H_2O. When a mixture of sodium phenylalanine (0.3 M) and ethyl acetoacetate (0.3 M) was incubated in H_2O at pH 9.5, the formation of enamine in aqueous solution was confirmed. The intensities of the characteristic ^{13}C-NMR signals due to C-4, C-5 and C-6 of the enamine thus formed in H_2O increased during the incubation.

Based on these findings together with the fact that the enamine is gradually hydrolyzed in the aqueous solution to afford phenylalanine and ethyl acetoacetate, we conclude that an equilibrium between the enamine and its constituents exists as shown in Chart 1.



[E]=enamine, [L]=phenylalanine, [M]=ethyl acetoacetate, $K=K_1K_2K_3K_4=[E][H_2O]/([L][M])$, $K'=K/[H_2O]$, $K_{obs}=[E]/([L_{total}][M])$, $[L_{total}]=[L]+[LH^+]$, $\log(K'/K_{obs})=pK_a-pH$, $K_a=[L][H^+]/[LH^+]$.

Chart 1. Equilibrium between Enamine and Its Hydrolysis Products

Discussion

It has been reported[6] that the formation of enamine in aqueous solution is very difficult, because the dehydration at the step 2 in Chart 1 is the limiting step for the enamine formation. However, the results obtained by UV-spectrophotometric and NMR studies in this work show that phenylalanine enamine of ethyl acetoacetate is easily formed even in aqueous solution under alkaline conditions. There is an equilibrium between the enamine and the hydrolyzed products with many intermediate steps as shown in Chart 1. The ^{1}H-NMR results in D_2O support the intermediate presented in step 1′ in Chart 1, because the hydrogens at C-4 and C-6 (see Table I) in the enamine were displaced with D.

The equilibrium constants at each step shown in Chart 1 can be written as follows:

$$K_1=[E][H^+]/[IH^+] \quad (1)$$

$$K_2=[IH^+][H_2O][DH^+] \quad (2)$$

$$K_3=[DH^+]/[D^\pm][H^+] \quad (3)$$

$$K_4=[D^\pm]/[L][M] \quad (4)$$

The equilibrium constant, K, between the enamine and its hydrolyzed products is given by Eq. 5.

$$K=K_1K_2K_3K_4 \quad (5)$$
$$=[E][H_2O]/[L][M]$$

In Eq. 5, $[H_2O]$ may be regarded as constant; thus Eq. 6 is obtained.

$$K'=K/[H_2O]=[E]/[L][M] \quad (6)$$

In this equation, K' does not show dependency on the hydrogen ion concentration of the aqueous solution, though K_{obs} does show such dependency.

From these findings, the behavior of phenylalanine in aqueous solution may be another factor influencing the observed equilibrium constant, K_{obs}. It may be considered that phenylalanine must have an unionized amino moiety if it is to interact with ethyl acetoacetate to form the enamine. The concentration of free phenylalaline having an unionized amino moiety, [L], in Eq. 6 can be calculated from K_a at the amino moiety.

$$K_a=[L][H^+]/[LH^+] \quad (7)$$

$$[L_{total}]=[L]+[LH^+] \quad (8)$$

where $[L_{total}]$ represents the total concentration of phenylalanine.

$$K_{obs}=[E]/[L_{total}][M] \quad (9)$$

$$1/K_{obs}=[L][M]/[E]+[LH^+][M]/[E] \quad (10)$$

$$=1/K'(1+[H^+]/K_a) \quad (11)$$

$$K'/K_{obs}-1=[H^+]/K_a \quad (12)$$

$$\log(K'/K_{obs}-1)=pK_a-pH \quad (13)$$

By using Eq. 13, the K' value can be estimated from K_{obs} and pK_a of phenylalanine, 9.24. The K' value obtained from Eq. 13 was not dependent on the pH of the aqueous solutions tested (Fig. 3). Since $K=K'[H_2O]$ from Eq. 6 and K' was constant, K should also be constant.

The above findings indicate that, although enamine formation in aqueous solution is not influenced by pH, the increase of the amino-ionized form of phenylalanine at pH below of the pK_a results in a decrease in the enamine formation. These findings lead us to conclude that

greater efficacy of adjuvant action of enamine may be achieved if the rectal pH can be kept alkaline.

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