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The Behavior of 1,4-Benzodiazepine Drugs in Acidic Media. V.¹⁾ Kinetics of Hydrolysis of Flutazolam and Haloxazolam in Aqueous Solution²⁾

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The hydrolysis of flutazolam and haloxazolam was investigated kinetically. The cleavage reaction of the diazepinone nucleus of flutazolam was reversible, and the ring-cleaved form was in equilibrium with the ring-closed form in aqueous solution. On the other hand, the cleavage reaction of haloxazolam was irreversible. It was concluded that the 2-hydroxyethyl substituent attached to the amide nitrogen atom of flutazolam is responsible for the reversible character of the hydrolytic cleavage of the diazepinone nucleus. The hydrolysis mechanism was elucidated on the basis of the pH-rate profile. To compare the effects of substituents on the hydrolysis, the rate constants of oxazolam and cloxazolam in acidic solution were also determined.

Keywords—flutazolam; haloxazolam; cloxazolam; oxazolam; benzodiazepine; hydrolysis; equilibrium reaction; kinetics; pH-rate profile; hydrogen bonding

In previous papers,³⁾ the authors discussed the structural changes, in acidic aqueous solution, of benzodiazepinooxazoles (oxazolam (IO), cloxazolam (IC), haloxazolam (IH), and flutazolam (IF)), as shown in Chart 1) chiefly on the basis of nuclear magnetic resonance (NMR) spectrometry. The authors suggested that among these drugs IO, IC, and IH^{3a)} (carrying no substituent at position 7) underwent irreversible hydrolytic cleavages of both the oxazolidine and benzodiazepine rings to give benzophenone-type compounds, whereas IF^{3b)} (carrying a 2-hydroxyethyl group at position 7) underwent a reversible ring-opening reaction.

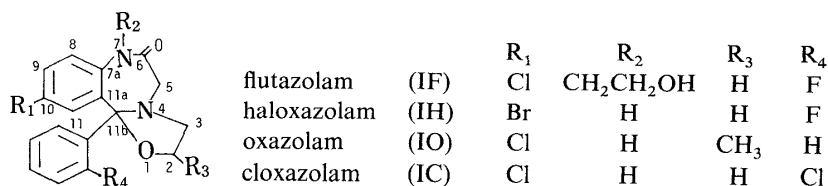


Chart 1

Ikeda and Nagai⁴⁾ recently reported the degradation kinetics of IO in aqueous solution in detail, but did not study other benzodiazepinooxazoles. A comparative study on the kinetics of hydrolysis among these drugs would be interesting because of the clear differences in the hydrolytic properties and rates.

In this paper, we describe the kinetics and mechanism of hydrolysis of IF and those of IH, which is structurally similar to IF. The effect of substituents on the hydrolysis is also discussed based on the hydrolytic rates of other benzodiazepinooxazoles in acidic aqueous solution.

Experimental

Materials—IF (Lot No. A 424490) and IH (Lot No. 10) were kindly supplied by Mitsui Pharmaceutical Co., Ltd. and Sankyo Co., Ltd., respectively, and were used without further purification. *N*-[4-Chloro-2-(2-fluorobenzoyl)]phenyl-*N*-(2-hydroxyethyl)-2-(2-hydroxyethyl)aminoacetamide hydrochloride (hydrolyzate of flutazolam, IIIF) and *N*-[4-bromo-2-(2-fluorobenzoyl)]phenyl-2-(2-hydroxyethyl)aminoacetamide (hydrolyzate of haloxazolam, IVH) isolated in previous studies³⁾ were used. *N*-Acetyl-2-amino-5-chlorobenzophenone (AcACB) and *N*-acetyl-*N*-methyl-2-amino-5-chlorobenzophenone (AcMeACB) were synthesized from 2-amino-5-chlorobenzophenone by procedures similar to those reported by Walker *et al.*⁵⁾ All other chemicals were obtained commercially and were of reagent grade.

Apparatus—Ultraviolet (UV) absorption spectroscopy was carried out with a Hitachi UV-124 spectrophotometer. The pH values were measured with a Hitachi-Horiba F-7_{LC} pH meter.

Kinetic Runs—The buffer systems used were as follows: below pH 1.5, an appropriate concentration of hydrochloric acid; pH 1.6–3.5, 0.05 M glycine-HCl; pH 4.0–5.5, 0.1 M acetate; pH 6.0–7.5, 0.03 M phosphate; pH 8.0–9.0, 0.025 M borate–0.05 M phosphate; pH 10.0–11.0, 0.025 M borate–0.025 M carbonate; pH 12.0, 0.025 M borate–0.05 M NaOH. The experiments were carried out at 25 °C in buffer containing 5% (v/v) ethanol with $\mu = 0.1$ (NaCl). When activation energies were determined, temperatures were controlled within ± 0.2 °C.

Although the effect of ethanol on the reaction rates is not clear at present, a stock solution of a sample (4×10^{-4} M) was prepared in ethanol for experimental convenience, that is, for easy solubilization of the drugs. The solution was diluted to 2×10^{-5} M with a buffer solution, and aliquots were withdrawn at appropriate intervals to measure the absorbance at a fixed wavelength (λ_{\max}).

The pseudo first-order rate constant (k_{obs}) was calculated from the slope of a linear plot of $\log(A_t \sim A_\infty)$ versus time, where A_t and A_∞ are the absorbance readings at time t and at infinity (when no further absorbance change occurred), respectively. When A_∞ was unknown, the Guggenheim method⁶⁾ was applied for the determination of the rate constants.

Results and Discussion

Hydrolysis of Flutazolam

Figure 1 shows the UV spectral change of IF with time in aqueous acidic solution (pH 1.2). The initial spectrum observed for the solution is attributable to an iminium structure

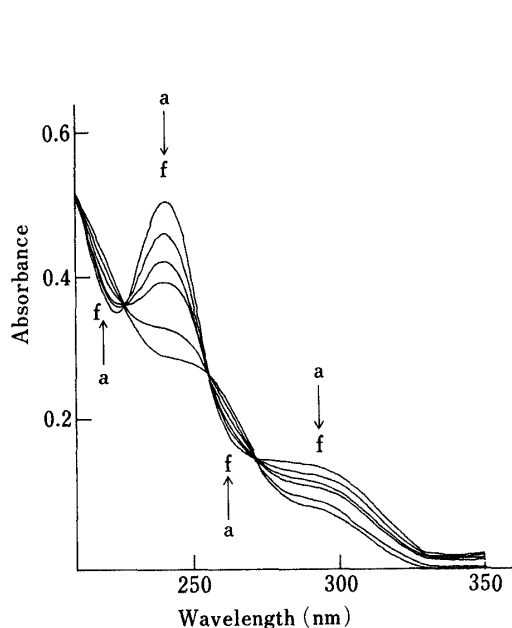


Fig. 1. UV Spectral Changes of Flutazolam (IF, 2.00×10^{-5} M) with Time at pH 1.2 and 25 °C

a, 0 min; b, 10 min; c, 20 min; d, 30 min; e, 60 min; f, 100 min.

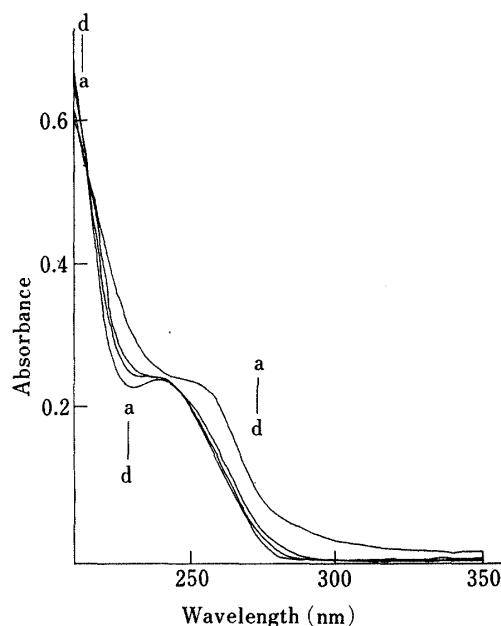


Fig. 2. UV Spectral Changes of Flutazolam (IF, 2.00×10^{-5} M) and Its Hydrolyzate (IIIF, 2.00×10^{-5} M) with Time at pH 7.4 and 25 °C

IF: d, 0 min; c, 120 min. IIIF: a, 0 min; b, 120 min.

(IIF, see Chart 2) cleaved in the oxazolidine ring in view of the fact that the apparent pK_a value ($-\log ([IF][H^+]/[IIF])$) for the reaction ($IIF \rightleftharpoons IF + H^+$) was 5.4.⁷⁻⁹ The reactions of $IIF \rightleftharpoons IF + H^+$ are considered to be very fast.⁹ Carbon-13 nuclear magnetic resonance (¹³C-NMR) spectral data also supported the form of IIF.^{3b} The UV spectrum of IIF changes

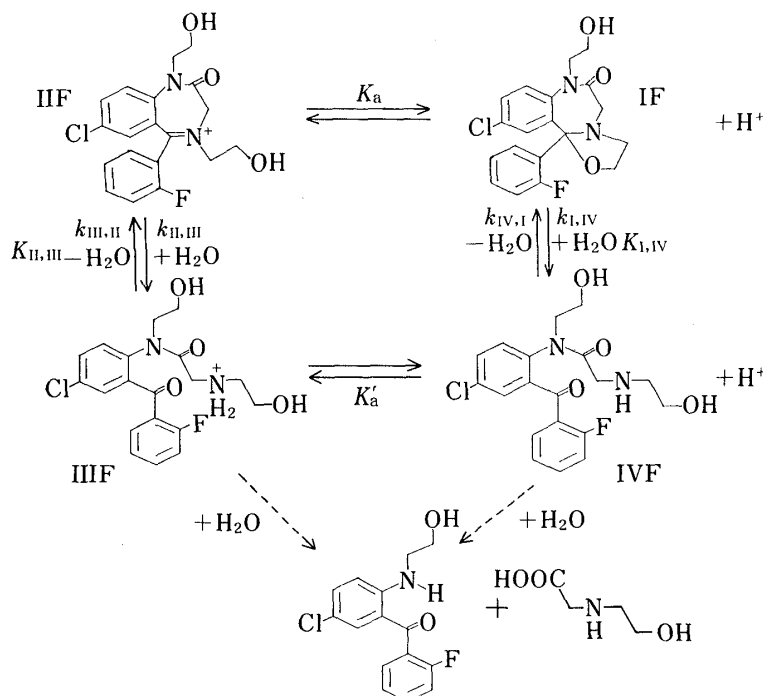


Chart 2

according to an apparent first-order one-step reaction with isosbestic points at about 227, 255 and 270 nm. The spectrum after completion of the change is in agreement with that of the hydrolyzate (IIIF) isolated previously^{3b} after cleavage of the benzodiazepine ring of IIF. Accordingly, the spectral change in the acidic solution corresponds to that from IIF to IIIF. Similar spectral changes were observed in buffer solutions of pH 3 and 5. In a weak acid solution (*e.g.*, pH 5.5) a very slight UV spectral change was obtained in the reaction started from IIIF (data not shown). At pH 5.5 the spectrum after 100 min of reaction initiated from IIIF was approximately in agreement with that of the reaction started from IIF. Accordingly, an equilibrium of IIF with IIIF, displaced toward the latter, is suggested to exist in the weak acid solution.

Figure 2 shows the UV spectral changes of IF and IVF in a weak alkaline solution (pH 7.4). When IF and IIIF are dissolved individually in the pH 7.4 buffer solution, the former shows the increase in absorbance at 230 nm, whereas the latter shows opposite spectral changes. Almost identical spectra were observed at the ends of the reactions (2 h later) regardless of the starting compound (IF or IIIF) and the pH of the alkaline buffer solution (pH 8.0 or 9.0), indicating that the equilibrium reactions occur as shown in Chart 2.

Hydrolysis of Haloxazolam

IH in the acidic buffer solution exhibited spectral changes with time (data not shown) similar to those of IF shown in Fig. 1. At the pH region above 7.4, the spectra of the IH reaction solution showed an isosbestic point at about 220 nm. The spectra of the acidic and alkaline solutions finally shifted to nearly the same curves, indicating that the same reaction products were present in both solutions. Furthermore, the spectra obtained by dissolving IVH itself in the above acid and alkaline solutions exhibited no change, and agreed with spectra

measured after the completion of the absorbance changes caused by the respective reactions. The hydrolysis of IH, therefore, takes place irreversibly in both solutions.

In chloroform-*d*, the hydrolyzate (IVH) of IH is assumed to exist in a *trans* conformation with respect to the aniline hydrogen and the amide carbonyl group, and to possess hydrogen bonding between the aniline hydrogen and the benzophenone carbonyl oxygen, forming a stable conformation.^{3a)} When the UV spectra of AcACB and AcMeACB shown in Chart 3 were compared with that of IVH in alkaline solution, the spectrum of IVH was similar to that of AcACB.¹⁰⁻¹³⁾ Therefore, IVH is suggested to have a stable conformation in aqueous solution as well as in the non-aqueous solution. No ring-closing reaction occurred, that is, the hydrolysis is irreversible, because the 2-hydroxyethyl amino group of IVH can not approach the benzophenone carbonyl group.

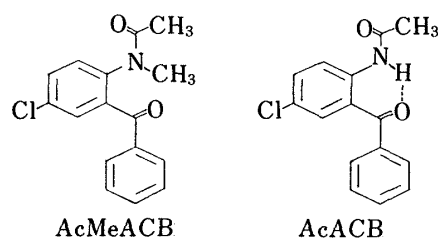


Chart 3

Rate Constants of the Hydrolysis

The reaction scheme in Chart 2 is intended to explain the equilibrium reactions of these molecular species. The constants $k_{II,III}$ and $k_{III,II}$ represent the rate constant of the benzodiazepine ring-opening reaction from the iminium form (IIF) and that of ring-closing from the hydrolyzate (IIIF), respectively. The constants $k_{I,IV}$ and $k_{IV,I}$ are the rate constants of ring-opening from IF and ring-closing from IVF, respectively. The constants $K_{II,III}$ and $K_{I,IV}$ represent the equilibrium constants in the acid and alkaline regions, respectively, and are defined as $k_{III,II}/k_{II,III}$ and $k_{IV,I}/k_{I,IV}$, respectively. K_a and K'_a are the apparent dissociation constants of IIF ($[IF][H^+]/[IIF]$) and IIIF ($[IVF][H^+]/[IIIF]$), respectively. The processes of amide hydrolysis (expressed by the notation “ $\text{---}\rightarrow$ ”) were negligible under the experimental conditions employed in this study because of the very slow rates as compared with the other process rates.

Because the pK_a value of IIF is 5.4,⁷⁾ IIF is negligible at pH 7.4 or above. Taking into account only two molecular species (IF and IVF), Eq. 1 is obtained when the reaction is started from IVF.

$$\frac{d[IF]}{dt} = k_{IV,I}[IVF]_0 - (k_{IV,I} + k_{I,IV})[IF] \quad (1)$$

where $[IVF]_0$ is the initial concentration of IVF. Equation 1 is integrated to give Eq. 2.

$$\log([IF]_{eq} - [IF]) = \frac{k_{IV,I} + k_{I,IV}}{2.303} \cdot t + \log[IF]_{eq} \quad (2)$$

where $[IF]_{eq}$ represents the equilibrium concentration of IF. Rearranging Eq. 2 by replacing the concentration term with the absorbance term gives Eq. 3.

$$\log(A_\infty \sim A_t) = \frac{k_{IV,I} + k_{I,IV}}{2.303} \cdot t + \log(A_\infty \sim A_0) \quad (3)$$

where A_0 is the absorbance of the reaction solution at zero time (see also the experimental section). The pseudo first-order rate constant (k_{obs}) determined experimentally, therefore,

corresponds to $k_{IV,I} + k_{I,IV}$. The equilibrium constant $K_{I,IV}$ is obtained from Eq. 4 in which the equilibrium concentrations of the respective molecular species are calculated by using the absorbance of the equilibrium solution, the molar absorptivities (ϵ in $M^{-1} cm^{-1}$) of the respective species (IF and IVF), and $[IVF]_0$.

$$K_{I,IV} = \frac{k_{IV,I}}{k_{I,IV}} = \frac{[IF]_{eq}}{[IVF]_{eq}} \quad (4)$$

The individual rate constants, $k_{IV,I}$ and $k_{I,IV}$, are estimated by using Eqs. 4 and 5.

$$k_{obs} = k_{IV,I} + k_{I,IV} = k_{I,IV}(K_{I,IV} + 1) \quad (5)$$

Around the apparent pK_a region (pH 5–7), the very small absorbance change caused by the reactions prevented the determination of an accurate k_{obs} value. The reason for the small change is probably as follows. The order of magnitude of the molar absorptivity is $\epsilon_{IIF} > \epsilon_{IIIF} \approx \epsilon_{IVF} > \epsilon_{IF}$ at about 235 and 300 nm, which are the wavelengths showing relatively large absorbance changes due to the reactions (see Figs. 1 and 2, the section on the hydrolysis of haloxazolam, and also Fig. 1(a) in our previous paper⁹). The reaction from IIF to IIIF causes a decrease in absorbance and that from IF to IVF, an increase in absorbance. The changes in absorbance cancel each other, and the resulting change due to the reactions is very small. In addition, the occurrence of the reverse reaction ($k_{IV,I}$) also may contribute to the small absorbance change.

Below pH 5.0, the k_{obs} value was obtained by ordinary first-order analysis.

The hydrolysis of IH followed first-order kinetics at any pH value. The rate constant was obtained from the slope of the plot.

pH Profile of the Rate Constant of Flutazolam

The logarithm of the rate constant k_{obs} (\circ) was plotted against the pH value of the solution (Fig. 3). When the k_{obs} value is divided into the rate constant of the ring-opening reaction ($k_{II,III}$ or $k_{I,IV}$) and that of the ring-closing reaction ($k_{III,II}$ or $k_{IV,I}$) using the above

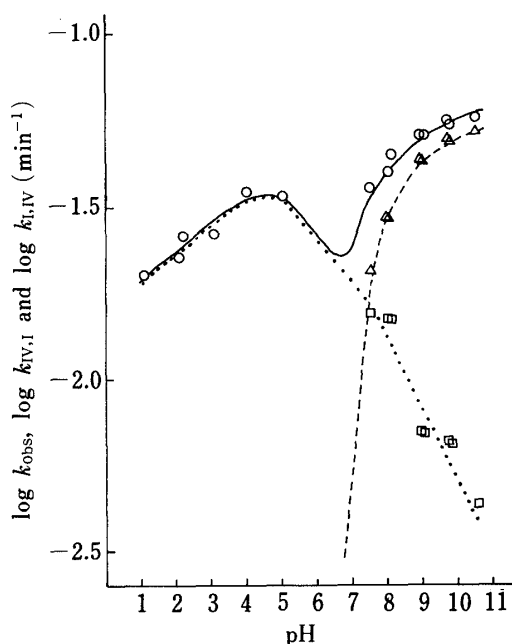


Fig. 3. pH-Rate Profile for the Hydrolysis of Flutazolam (IF) at 25°C

\circ , k_{obs} ; \triangle , $k_{IV,I}$; \square , $k_{I,IV}$.

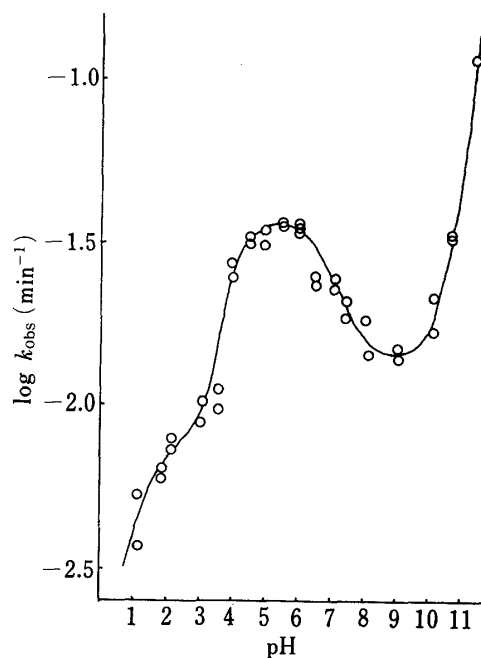


Fig. 4. pH-Rate Profile for the Hydrolysis of Haloxazolam (IH) at 25°C

analytical method, in the acid region the product of the molar absorptivity of IIIF and the initial concentration of IF used (*i.e.*, IIF) was equal to the absorbance of the equilibrium solution. Therefore, IIIF is greatly predominant in the acid equilibrium solution, *i.e.*, in the equilibrium of IIF with IIIF. Since $k_{II,III}$ is nearly equal to k_{obs} in the acid region and since in the alkaline region the $\log k_{I,IV}$ values (\square) appear to decrease linearly with the pH values, a dotted line between pH 5.0 and 7.4 (which could not be determined experimentally) was drawn by connecting k_{obs} at pH 5.0 to $k_{I,IV}$ at pH 7.4.

The constants $k_{IV,I}$ and $k_{III,II}$ are involved in the ring-closing reaction. The $\log k_{IV,I}$ -pH profile (\triangle) and also the very small value of $k_{III,II}$ lead to Eq. 6 relating $k_{IV,I}$ to the hydrogen ion concentration $[H^+]$.

$$k_{IV,I} = \frac{k_{IV,I}^0 K'_a}{[H^+] + K'_a} \quad (6)$$

where $k_{IV,I}^0$ is the intrinsic ring-closing rate constant. From a plot of $1/k_{IV,I}$ against $[H^+]$, $k_{IV,I}^0$ and K'_a were estimated as 5.0×10^{-2} (min^{-1}) and 1.1×10^{-8} (M, $\text{p}K'_a = 8.0$), respectively. The broken curve in Fig. 3 was calculated by using Eq. 6 after the substitution of these values.

The solid curve between pH 5.0 and 7.4, which was experimentally indeterminable, was estimated from the dotted curve (based on $k_{I,IV}$ and $k_{II,III}$) and the broken curve (based on $k_{IV,I}$ and $k_{III,II}$), since k_{obs} is the sum of the rate constant for ring-opening and that for ring-closing. These analyses of the pH-rate profiles indicate that the ring-opening and the ring-closing reactions of flutazolam take place predominantly in the acid and alkaline regions, respectively.

pH Profile of the Rate Constant of Haloxazolam

Figure 4 gives the pH-rate profile of IH. Because the hydrolysis of IH is irreversible, k_{obs} itself is the hydrolysis rate constant. The effect of buffer concentration on k_{obs} is not corrected for in the present experiment. However, a pH-rate profile (not shown here) made by extrapolating the reported data for IO⁴) to the present buffer concentration conditions closely resembled the pH-rate profile of IH shown in Fig. 4. For this reason, as described by Ikeda and Nagai,⁴) IH appears to exhibit kinetic properties similar to those of IO (see ref. 4).

Comparison of Hydrolysis Rates of Benzodiazepinooxazoles and Temperature Dependence of the Rates

The rate constants of IC and IO were also obtained at various temperatures, to compare the effect of substituents on the hydrolysis rate. The constants are summarized in Table I along with the values of activation energies calculated from the Arrhenius plot.

When the values of k_{obs} for these drugs are compared at 37 °C, a bromine atom at position 10 of IH appears to exert effect, nearly equal to a chlorine atom, on the iminium carbon atom. The hydrolysis of IH would be slightly promoted as compared with that of IO

TABLE I. Rate Constants^{a)} and Activation Energies^{b)} in Hydrochloric Acid Solution (pH 1.3)

Drugs	Temp. (°C)	k_{obs} (h^{-1})					Activation energies kcal/mol
		13	24	37	46	56	
Flutazolam (IF)		0.422	1.10	3.44	—	—	15.4
Haloxazolam (IH)		—	0.388	0.972	2.12	—	15.2
Oxazolam (IO)		—	0.167	0.626	1.61	—	18.6
Cloazolam (IC)		—	—	0.044	0.102	0.221	17.3

a) Average of two determinations. b) Calculated by means of the Arrhenius equation.

bearing no substituent near the iminium site which undergoes hydrolysis. Presumably the electronegativity of the fluorine atom at position 2' (R_4) makes the iminium carbon atom electron-deficient. On the other hand, the hydrolysis of IC is remarkably slower than that of IO. This cannot be explained in terms of the electronegativity of the chlorine atom at position 2' but may be explained by the steric effect of the chlorine atom,¹⁴⁾ because the effect of the electronegativity of substituents at position 2' on the rate was small (see the comparison between IH and IO above). The hydrolytic rate of IF is greater than that of IH, although both compounds carry the fluorine substituent at position 2', and this may be because the distortion of the benzodiazepine ring resulting from the 2-hydroxyethyl group substituted at the amide nitrogen atom affects the hydrolysis.

The value of activation energy of IO was the greatest and that of IC was somewhat smaller. The activation energy of IH was nearly equal to that of IF and these values were the smallest among these compounds.

It is reported that the ring-cleaved compounds carrying no substituent on the amide nitrogen atom have inferior pharmacological activity *in vivo* as compared to the parent compounds.¹⁵⁾ On the other hand, the ring-cleaved compound of IF carrying a substituent has activity nearly equal to that of the parent compound.^{3b)} These phenomena may be explained reasonably in terms of the results of this study as follows. The pharmacological activities of these drugs are due to the benzodiazepine ring-closed forms. The drugs orally administered would undergo partial cleavage of the benzodiazepine ring. The compounds carrying no substituent on the amide nitrogen atom (*e.g.*, IH) do not regenerate the parent compounds under weak alkaline conditions in the intestine. On the other hand, a compound such as IF undergoes a ring-closing reaction and regenerates the parent compound in the intestine.

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