[Chem. Pharm. Bull. 29(12)3671—3679(1981)]

The Stability of Carboquone in Aqueous Solution. I. Kinetics and Mechanisms of Degradation of 2,5-Diethylenimino-1,4-benzoquinone in Aqueous Solution

Akira Kusai,* Seiji Tanaka, and Seigo Ueda

Product Development Laboratories, Sankyo Co., Ltd., Hiromachi, Shinagawa-ku, Tokyo, 140, Japan

(Received May 18, 1981)

The kinetics and mechanisms of the degradation of 2,5-diethylenimino-1,4-benzoquinone (EB) in aqueous solution were investigated as a function of pH (4—11), temperature (20—50°C), buffer concentration (0.01—0.1 M) and ionic strength (0.1—0.5) by means of high pressure liquid chromatography.

The reaction followed pseudo first-order kinetics. The pH-rate profile showed slopes of −1 at pH below 7.5 and +1 at pH over 7.5. Thus, the degradation of EB is subjected to specific acid-base catalysis. No effect of buffer concentration on the degradation rate of EB was apparent in the range of pH 4—11, though some effect of ionic strength on it was observed below pH 7. The apparent activation energies for the degradation of EB at pH 4 or 5 and pH 10 or 11 were 14 and 19 kcal/mol, respectively. In the range of pH 4—6, EB was degraded to 2,5-diethanolamino-1,4-benzoquinone with sequential hydrolytic cleavage of two ethylenimine rings. In the range of pH 10—11, EB was degraded to 2,5-dihydroxy-1,4-benzoquinone with sequential substitution of the two ethylenimine rings by hydroxyl ion (radical). In the range of pH 7—9, EB was degraded to 2,5-diethanolamino-1,4-benzoquinone, 2,5-dihydroxy-1,4-benzoquinone and 2-ethanolamino-5-hydroxy-1,4-benzoquinone by the two mechanisms described above.

Keywords——ethylenimino benzoquinone; degradation kinetics and mechanisms; hydrolytic ring cleavage; Michael reaction; HPLC

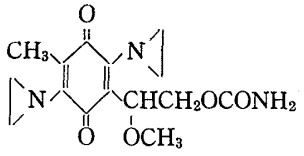
Carboquone [CQ; 2,5-bis(1-aziridinyl)-3-(2-carbamoyloxy-1-methoxyethyl)-6-methyl-1,4-benzoquinone][1,2] is one of the antitumor agents with ethylenimino-benzoquinone structure (Chart 1) clinically classified as alkylating agents. It is known that CQ is relatively unstable in aqueous solutions, but its degradation mechanism and degradation products have remained obscure. In order to obtain a well defined pharmaceutical dosage form of CQ and to consider the biotransformation of CQ administered orally or parenterally, it is necessary that some information be obtained on the degradation mechanism of CQ in aqueous solution.

It is generally thought that the instability of CQ is mainly due to lability of the two ethylenimine groups in the structure. There are many reports on the degradation mechanism of ethylenimine compounds,[3–5] but only a few on that of ethylenimino-benzoquinone compounds. Anhalt *et al.*[6] potentiometrically studied the degradation of 2,5-diethylenimino-3,6-diisopropoxy-1,4-benzoquinone. This compound was degraded to the same compound in both acidic and basic aqueous solution ,*i.e.* 2,5-diethanolamino-3,6-diisopropoxy-1,4-benzoquinone with 2-ethylenimino-5-ethanolamino-3,6-diisopropoxy-1,4-benzoquinone as an intermediate.

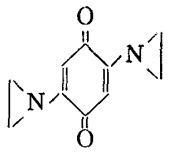
On the other hand, a preliminary study revealed that high pressure liquid chromatography HPLC patterns for aged CQ aqueous solutions were complex and considerably different in acidic and basic media.

These results suggest a more complex degradation mechanism for CQ, in contrast to the report of Anhalt *et al.*[6] A more definite analysis of the degradation mechanism of CQ on the basis of this preliminary study, however, has been precluded by the appearance of a large number of HPLC peaks and by the complex structure of the molecule.

Therefore, 2,5-diethylenimino-1,4-benzoquinone (EB, Chart 1) was selected as a model compound for the study of CQ degradation in aqueous solution because of its simple structure



Carboquone



EB

Chart 1

and because the kinetics and mechanisms of degradation of EB in aqueous solution could be conveniently investigated by the HPLC technique.

Experimental

Materials——EB was prepared according to Gauss *et al.*[7] (mp 203°C). *Anal.* Calcd for $C_{10}H_{10}N_2O_2$: C, 63.14; H, 5.30; N, 14.73. Found: C, 62.83; H, 5.29; N, 14.70.

2,5-Diethanolamino-1,4-benzoquinone (EAB) was prepared as follows. Ethanolamine (0.6 ml) was added dropwise with stirring to a solution of 1g of *p*-benzoquinone in 40 ml of 50% ether in ethanol at room temperature. The resulting mixture was allowed to stand in a refrigerator for 2 d. The separated crystals were collected and recrystallized from ethanol to give 0.3 mg of EAB (mp 266°C). *Anal.* Calcd for $C_{10}H_{14}N_2O_2$: C, 53.09; H, 6.24; N, 12.38. Found: C, 52.71; H, 6.16; N, 12.21.

2,5-Dihydroxy-1,4-benzoquinone (HB) was purchased from Aldrich Chemical Company, Inc.,[8] and recrystallized from ethanol (mp 220°C).

Other chemicals used were of the highest grade commercially available.

Buffer Solutions for Kinetic Study——Acetate buffer (pH 4 and 5), phosphate buffer (pH 6—8), borate buffer (pH 9) and carbonate buffer (pH 10 and 11) were used. The concentrations of buffer solutions were 0.01 M unless otherwise stated. The ionic strength was adjusted to 0.2 with sodium sulfate[9] unless otherwise stated.

Procedure for Kinetic Study——(a) EB Stock Solution: Two hundred and twenty-five milligrams of EB was dissolved in N,N-dimethyl acetamide and brought up to 100 ml.

(b) Degradation Kinetics of EB: One milliliter aliquots of EB stock solution were each mixed with 49 ml of buffer solution preincubated in a water bath thermostated at 20, 30, 40 or 50±0.1°C. At regular intervals, 5 ml of the sample solution was taken into a centrifugal tube with a stopper containing 10 ml of chloroform and shaken vigorously. After centrifugation at 3000 *g* for 5 min, 5 ml of the chloroform layer was pipetted into a pear-shaped flask and a drop of macrogol 400 was added.[10] The chloroform was evaporated, off and the residue was dissolved in 1 ml of methyl salicylate solution (0.2% in methanol) as an internal standard. The solution thus obtained was subjected to HPLC (Method I).

(c) Disappearance of EB and Appearance of the Degradation Products in Acidic Aqueous Solution: Each 1 ml aliquot of EB stock solution was mixed with 49 ml of buffer solution (salt-free) preincubated in a water bath thermostated at 40°C. At regular intervals, 5 ml of the sample solution was taken into a volumetric flask of 20 ml. Next, 1 ml of 0.5 M triethanolamino-acetate buffer solution of pH 7 to stop the reaction and 2 ml of methyl salicylate solution (0.1% in methanol) were added, and the whole was diluted to the mark with methanol. The solution thus obtained was analyzed by means of HPLC (Method I).

(d) Disappearance of EB and Appearance of Degradation Products in Basic Aqueous Solution: One milliliter aliquots of EB stock solution were each mixed with 49 ml of buffer solutions preincubated in a water bath thermostated at 40°C. Aliquots of 2 ml taken at regular intervals were each mixed with 0.5 ml of 0.5 M sodium bicarbonate to stop the reaction, 2 ml of chloroform was added and the whole was shaken vigorously. The chloroform layer was assayed spectrophotometrically for EB at a wavelength of 333 nm. One milliliter of the aqueous layer was mixed with 1 ml of 0.5% sodium salicylate aqueous solution as an internal standard. The solution thus obtained was analyzed by means of HPLC (Method II).

HPLC——Chromatography was performed on a Hitachi liquid chromatograph, model 635, equipped with a monitoring system operating at a wave length of 325 nm.

Method I: A column (5 mm i.d.×50 cm) of a porous styrene-divinylbenzene copolymer[11] was used. The mobile phase consisted of 95% (v/v) methanol and 5% (v/v) water containing 0.01 M sodium bicarbonate. The temperature was 40°C and the flow rate was 2 ml/min.

Method II: A column (4 mm i.d.×15 cm) of an octadecylsilane chemically bonded to totally porous silica gel[12] was used. The mobile phase was 0.05 M sodium bicarbonate in water containing 5% (v/v) methanol. The temperature was 50°C and the flow rate was 1 ml/min.

Results and Discussion

Degradation Kinetics of EB

Typical HPLC patterns of EB solutions stored under acidic and basic conditions are shown in Fig. 1. It is obvious that these HPLC patterns are significantly different from each other, and this suggests that the degradation mechanism of EB is different under acidic and basic conditions. In any case, semilogarithmic plots of the concentration of EB against time were linear and it was apparent that the degradation of EB follows pseudo first-order kinetics over a wide range of pH. Ionic strength only affected the degradation rate of EB at pH values below 7, as shown in Fig. 2. This also suggests that the degradation mechanism of EB is different under low and high pH conditions.

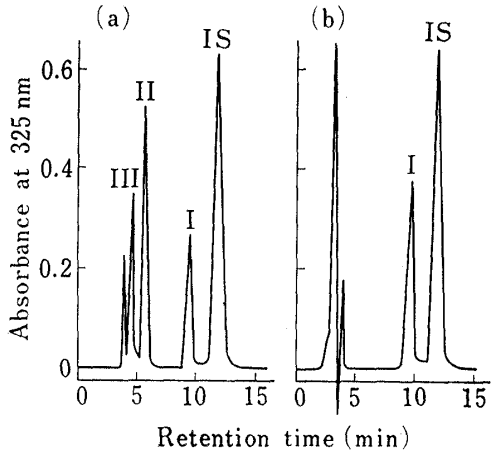


Fig. 1. Chromatograms obtained from Aqueous Solutions of EB by HPLC (Method I)

(a) from 0.01 M acetate buffer (salt-free), pH 4, after 2 min at 40°C. The remaining EB is 43%.
(b) from 0.01 M carbonate buffer (salt-free), pH 11 after 2 min, at 40°C. The remaining EB is 56.5%.
I : EB
II : 2-ethanolamino-5-ethylenimino-1,4-benzoquinone
III: 2,5-diethanolamino-1,4-benzoquinone (EAB)
IS : methyl salicylate (internal standard)

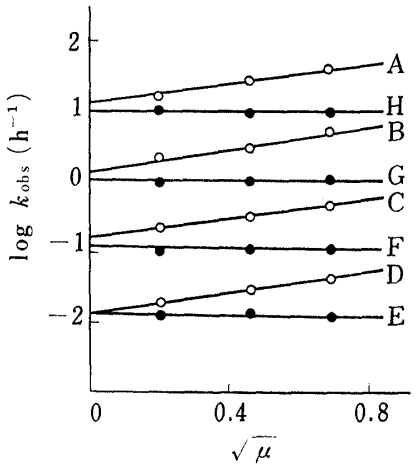


Fig. 2. The Effect of Ionic Strength on the Degradation Rate of EB at 30°C

A: pH 4, E: pH 8,
B: pH 5, F: pH 9,
C: pH 6, G: pH 10,
D: pH 7, H: pH 11.

No significant effect of the concentration of buffers on the degradation rate of EB was observed, as shown in Fig. 3.

By extrapolating the data obtained to zero ionic strength, a log k_o-pH profile was obtained. As shown in Fig. 4, plots of the rate constants gave a minimum at pH near 7.5. The slopes at pH below 7.5 and pH over 7.5 were -1 and $+1$, respectively. This result indicates that the degradation of EB is subject to specific acid-base catalysis.

The temperature dependency of the degradation rate of EB was estimated by Arrhenius plots of log k_{obs} *vs.* $1/T$. As shown in Fig. 5, the plots gave straight lines and the apparent activation energies calculated from the slopes of these lines at pH 4 or pH 5 and pH 10 or pH 11 were 14 and 19 kcal/mol, respectively.

Degradation Mechanism of EB in Acidic Aqueous Solution

Fig. 1(a) shows a typical HPLC pattern of EB solution stored at acidic pH. By comparing the retention times of the peaks, it was confirmed that peak I represented EB, and peaks II and III corresponded to the degradation products of EB.

The retention time and UV spectrum of peak III component fractionated in HPLC coincided with those of authentic EAB. At pH 4, 40°C, accompanying the decrease of peak

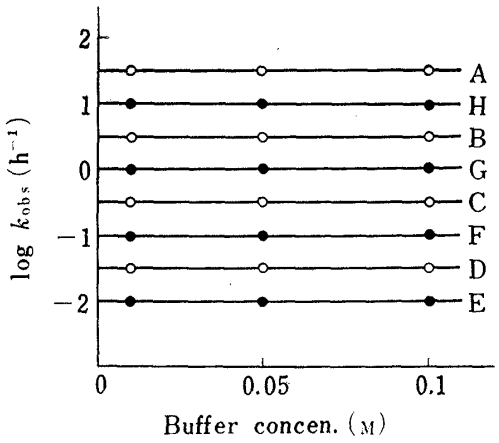


Fig. 3. The Effect of Buffer Concentration on the Degradation Rate of EB at 30°C and $\mu=0.2$

The ionic strength of 0.1 M phosphate buffer at pH 8 is over 0.2 (*i.e.* 0.27), but the data are included in the figure because no effect of ionic strength on the degradation rate was observed at this pH, as shown in Fig. 2.

A: acetate buffer, pH 4, E: phosphate buffer, pH 8,
B: acetate buffer, pH 5, F: borate buffer, pH 9,
C: phosphate buffer, pH 6, G: carbonate buffer, pH 10,
D: phosphate buffer, pH 7, H: carbonate buffer, pH 11.

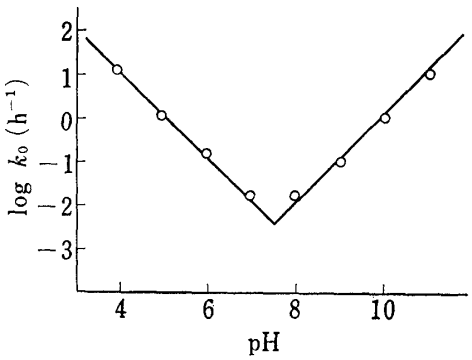


Fig. 4. Log k_0-pH Profile of EB Degradation at 30°C obtained by Extrapolating the Data to $\mu=0$

I, peak II increased gradually to attain a maximum at around 2 min, but later decreased with time, while peak III increased gradually after a short lag time and at times beyond 8 min no peaks other than peak III were observed on the chromatograms.

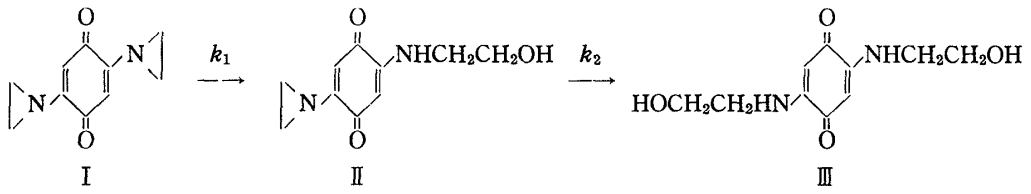


Chart 2

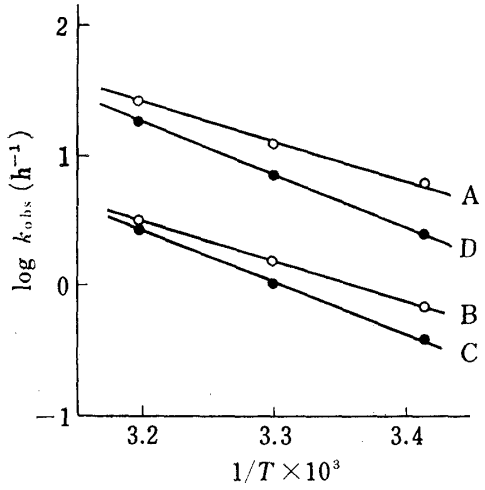


Fig. 5. Arrhenius Plots of Log k_{obs} at $\mu=0.2$

A: pH 4, B: pH 5, C: pH 10, D: pH 11.

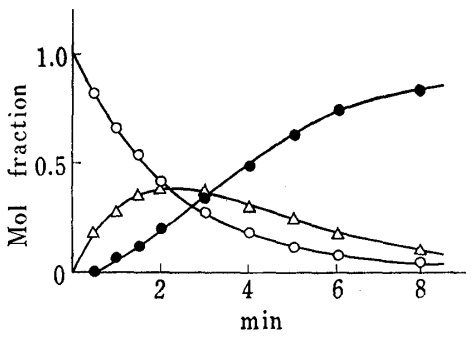


Fig. 6. Time Courses for I(○), II(△) and III (●) during EB Degradation at pH 4, 40°C

The lines are values calculated from equations 1, 2′ and 3′ with $k_1=k_2=25.6$ h^{-1}, obtained by trial-and-error fitting.

These results suggest that the peak II component is an intermediate in the formation of the peak III component (*i.e.* EAB), and the degradation mechanism of EB is proposed to be as shown in Chart 2. When EB is degraded according to Chart 2, the mol fractions of I, II and III at time t can be calculated from equations 1, 2 (or 2′) and 3 (or 3′), respectively,[13]

$$X(I_t) = \exp(-k_1 t) \qquad \text{eq. 1}$$

$$X(II_t) = \frac{k_1}{k_2 - k_1}\{\exp(-k_1 t) - \exp(-k_2 t)\} \qquad \text{eq. 2}$$

$$X(III_t) = 1 + \frac{1}{k_1 - k_2}\{k_2 \exp(-k_1 t) - k_1 \exp(-k_2 t)\} \qquad \text{eq. 3}$$

When k_1 is equal to k_2,

$$X(II_t) = k_1 t \exp(-k_1 t) \qquad \text{eq. 2'}$$

$$X(III_t) = 1 - \{\exp(-k_1 t) + k_1 t \exp(-k_1 t)\} \qquad \text{eq. 3'}$$

On the other hand, the mol fractions of I and III at time t can be obtained from HPLC data. The mol fraction of II can be calculated from equation 4, as follows,

$$X(II_t) = 1 - \{X(I_t) + X(III_t)\} \qquad \text{eq. 4}$$

Fig. 6 shows that the calculated values agreed well with observed values at pH 4, 40°C. At pH 5 and pH 6, similar agreements were observed between both values, assuming $k_1 = k_2 =$ 3.2 h^{-1} and 0.36 h^{-1}, respectively. Thus it is reasonable to consider that the peak II component is 2-ethanolamino-5-ethylenimino-1,4-benzoquinone.

From these data, it is apparent that EB is degraded to EAB with sequential hydrolytic cleavage of the two ethylenimine rings (Chart 2).

In this mechanism of degradation of EB, the initial step of the reaction is thought to be protonation of the trivalent nitrogen in the ethylenimine rings to yield the iminium ion (Chart 3).[3-5]

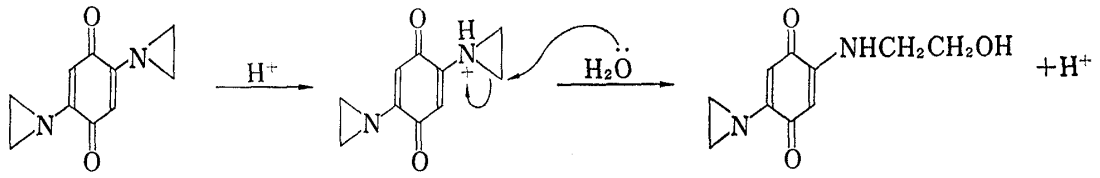


Chart 3

Degradation of EB in Basic Aqueous Solution

As mentioned above, the HPLC (Method I) patterns of EB solutions stored under acidic conditions and under basic conditions were quite different; under basic conditions, peak I (corresponding to EB) was observed, but peaks II and III observed in acidic solutions were not seen, and another peak(s) appeared just before the solvent peak (Fig. 1). Therefore, in order to obtain better resolution, another analytical procedure and different HPLC conditions (Method II) were employed.

When the sample solution was shaken with chloroform, EB was completely extracted into chloroform. Thus, the concentration of EB was determined spectrophotometrically by assaying the chloroform layer. The residual aqueous layer, when subjected to HPLC (Method II), gave two peaks, IV and V, as shown in Fig. 7, and the components of these peaks are believed to be degradation products of EB. Peak V component was considered to be HB, since the HPLC retention time and the UV spectrum of peak V component fractionated in HPLC coincided well with those of the authentic compound. At pH 11, 40°C, accompanying the rapid decrease of EB, peak IV component increased rapidly during the initial stage of the

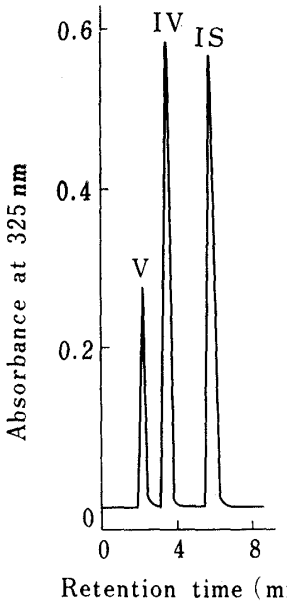


Fig. 7. Chromatogram obtained from Aqueous Solution of EB by HPLC (Method II)

From 0.01 M carbonate buffer (salt-free), pH 11, after 60 min at 40°C followed by chloro form treatment. The remaining ratio of EB is 0%.
IV: 2-ethylenimino-5-hydroxy-1,4-benzoquinone
V : 2,5-dihydroxy-1,4-benzoquinone (HB)
IS : sodium salicylate (internal standard)

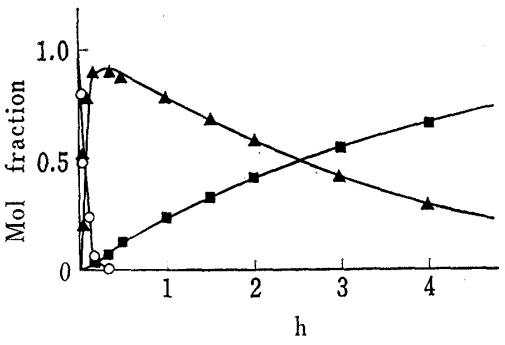


Fig. 8. Time Courses for I (○), IV (▲) and V(■) during EB Degradation at pH 11, 40°C

The lines were calculated from equations 5, 6 and 7 with $k_3=17.5\ h^{-1}$ and $k_4=0.281\ h^{-1}$ obtained by trial-and-error fitting.

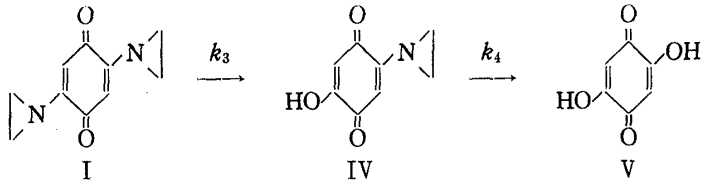


Chart 4

reaction and later decreased gradually, while the peak V component gradually increased after a short lagtime.

These results suggest that the peak IV component is an intermediate in the formation of the peak V component (*i.e.* HB), and the degradation mechanism of EB is proposed to be as shown in Chart 4. When EB is degraded according to Chart 4. The mol fractions of I, IV and V at time t can be calculated from equations 5, 6 and 7, respectively,[13]

$$X(I_t) = \exp(-k_3 t) \qquad \text{eq. 5}$$

$$X(IV_t) = \frac{k_3}{k_4 - k_3}\{\exp(-k_3 t) - \exp(-k_4 t)\} \qquad \text{eq. 6}$$

$$X(V_t) = 1 + \frac{1}{k_3 - k_4}\{k_4 \exp(-k_3 t) - k_3 \exp(-k_4 t)\} \qquad \text{eq. 7}$$

Also, the mol fractions of I and V at time t can be obtained from spectrophotometric determinations of the chloroform layers and from the HPLC data, respectively, using authentic compounds as standards. The mol fraction of IV can then be calculated from equation 8,

$$X(IV_t) = 1 - \{X(I_t) + X(V_t)\} \qquad \text{eq. 8}$$

As shown in Fig. 8, the calculated values agree well with the observed values at pH 11, 40°C. At pH 10, similar agreement was observed between both values, assuming $k_3=2.9\ h^{-1}$ and

k_4=0.043 h^{-1}. Thus it is reasonable to consider the peak IV component to be 2-ethylenimino-5-hydroxy-1,4-benzoquinone.

From these results it is apparent that EB is degraded to HB with sequential substitution of the two ethylenimine rings by hydroxyl ion (radical), and the initial step of the reaction is based upon the Michael reaction,[14] as shown in Chart 5.

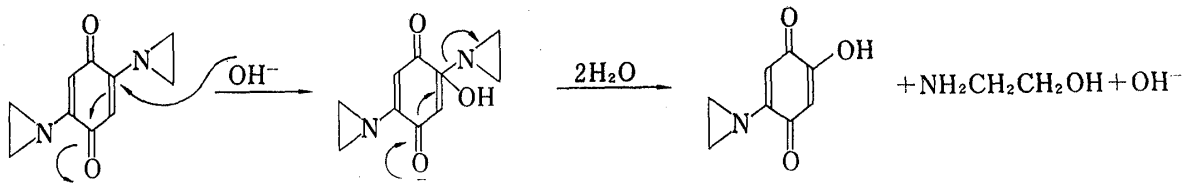


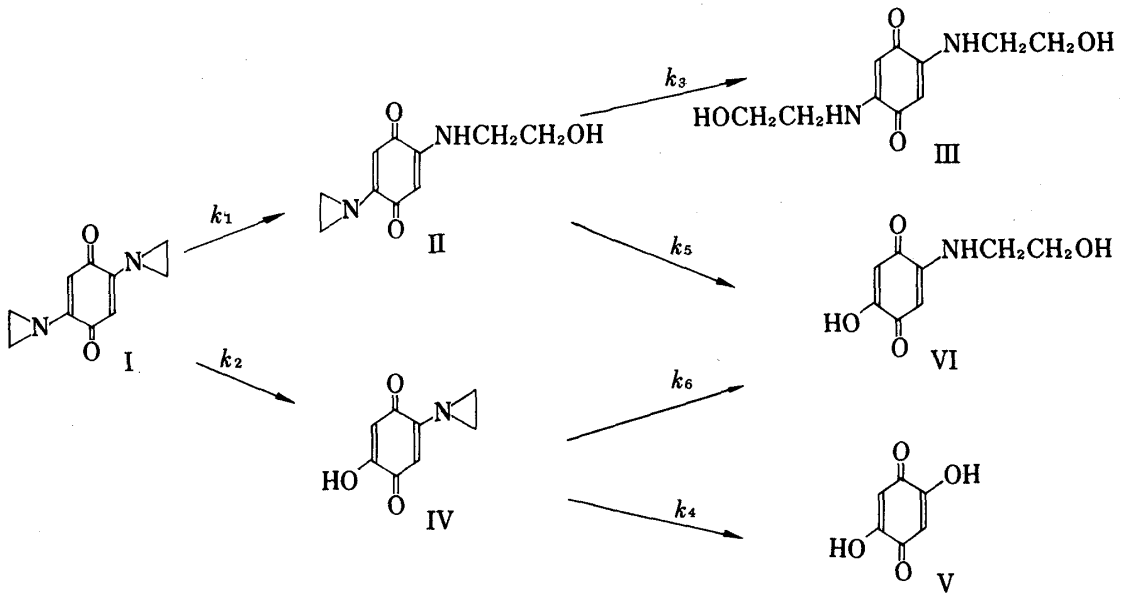
Chart 5

Although k_2 is equal to k_1, k_4 is much smaller than k_3. In the former case, the protonation and hydrolysis rates of the ethylenimine ring may be unaffected (of equally affected) by the p-substituent (*i.e.* ethylenimine or ethanolamine). In the latter case, however, the hydroxyl group produced in the first step (k_3) may form a resonance structure with the adjacent C=O group and this structure may reduce the rate of the second step (k_4).

Degration Mechanism of EB in Aqueous Solution of pH 7—9

For the sample solutions in the range of pH 7—9, Methods I and II of HPLC were both applied. As shown in Fig. 9, HPLC patterns of the sample solutions according to method I were the same as those of acidic solutions (*i.e.* peaks I, II and III were observed). However, HPLC of the sample solutions according to Method II gave an additional peak VI besides peaks IV and V.

Since peak VI appeared on acidifying the solution containing the peak IV component[15] with acetic acid, the peak VI component was considered to be 2-ethanolamino-5-hydroxy-1,4-benzoquinone. At pH 7, the peak II and III components were dominant and the peak VI component was minor, while at pH 8, the peak IV and VI components were dominant and the peak II, III and V components were minor, and at pH 9, no peaks other than peaks IV, V and VI were observed.



Cahrt 6

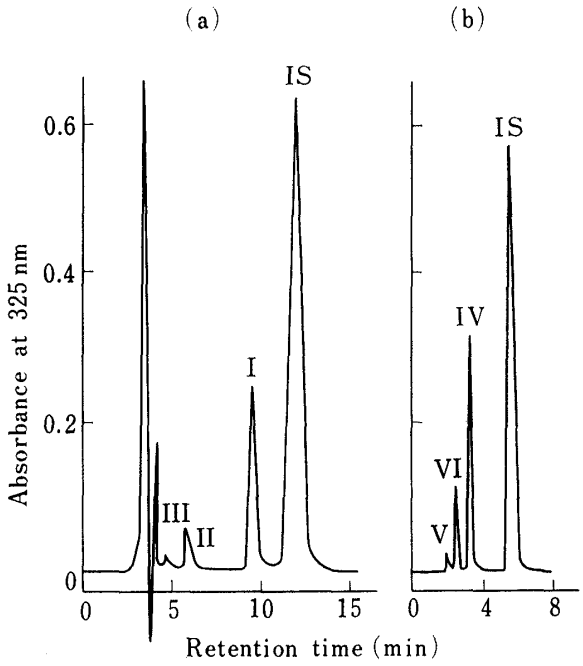


Fig. 9. Chromatograms obtained from 0.01 M Phosphate Buffer Solution of EB at pH 8 (Salt-free) after 24 h at 40°C by HPLC Method I (a) and Method II(b)

The remaining ratio of EB is 38%.
I : EB,
II : 2-ethanolamino-5-ethylenimino-1,4-benzoquinone,
III: 2,5-diethanolamino-1,4-benzoquinone (EAB),
IV: 2-ethylenimino-5-hydroxy-1,4-benzoquinone,
V : 2,5-dihydroxy-1,4-benzoquinone (HB),
VI: 2-ethanolamino-5-hydroxy-1,4-benzoquinone,
IS : (a) methyl salicylate, (d) sodium salicylate.

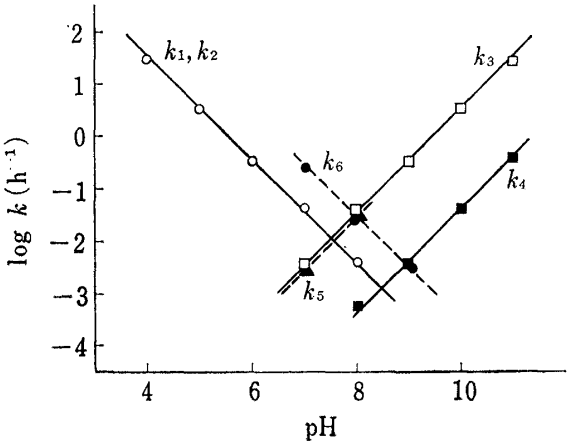


Fig. 11. Log k–pH Profile for Each Rate Constant at 40°C

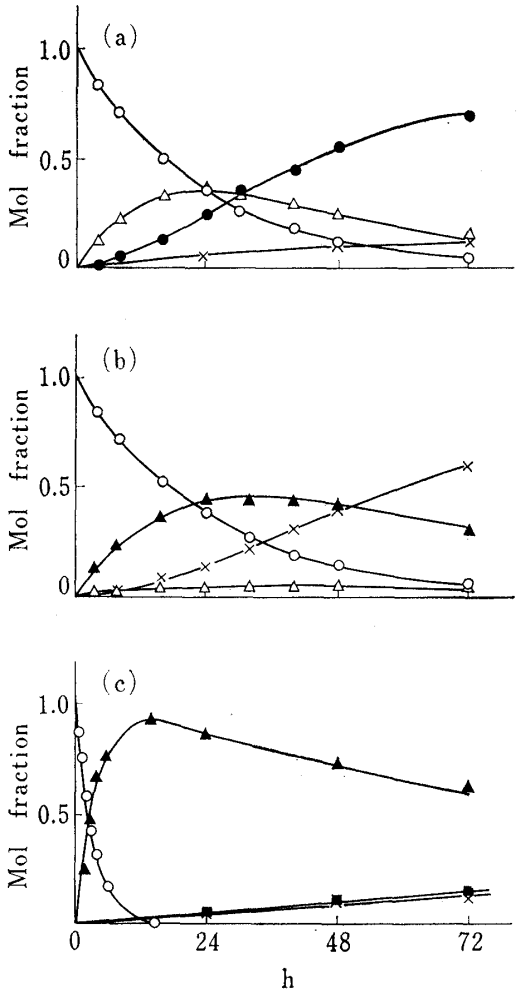


Fig. 10. Time Courses for I (○), II (△), III (●), IV (▲), V (■) and VI (×) during EB Degradation at (a) pH 7, 40°C, (b) pH 8, 40°C and (c) pH 9, 40°C

The lines were calculated from equations 9—14 with the following rate constants obtained by trial-and-error fitting.
(a) $k_1=k_2=0.041$, $k_3=k_5=0.003$, $k_4=0.36$ h^{-1} (k_6 was neglected).
(b) $k_1=k_2=0.004$, $k_3=k_5=0.036$, $k_4=0.0006$, $k_6=0.02$ h^{-1}.
(c) $k_3=0.279$, $k_4=k_6=0.0035$ h^{-1} (k_1, k_2 and k_5 were neglected).

These results suggest that EB is degraded in the range of pH 7—9 according to Chart 6 which is a combination of Chart 2 and Chart 4.

When EB is degraded according to Chart 6, the mol fractions of I, II, III, IV, V and VI at time t can be calculated from equations 9—14, respectively,

$$X(I_t) = \exp(-(k_1+k_3)t) \qquad \text{eq. 9}$$

$$X(II_t) = \frac{k_1}{(k_2+k_5)-(k_1+k_3)}\{\exp(-(k_1+k_3)t)-\exp(-(k_2+k_5)t)\} \qquad \text{eq. 10}$$

$$X(III_t) = \frac{k_1k_2}{(k_2+k_5)-(k_1+k_3)}\left[\frac{1}{k_1+k_3}\{1-\exp(-(k_1+k_3)t)\}\right.$$
$$\left.-\frac{1}{k_2+k_5}\{1-\exp(-(k_2+k_5)t)\}\right] \quad \text{eq. 11}$$

$$X(IV_t) = \frac{k_3}{(k_4+k_6)-(k_1+k_3)}\{\exp(-(k_1+k_3)t)-\exp(-(k_4+k_6)t)\} \quad \text{eq. 12}$$

$$X(V_t) = \frac{k_3k_4}{(k_4+k_6)-(k_1+k_3)}\left[\frac{1}{k_1+k_3}\{1-\exp(-(k_1+k_3)t)\}\right.$$
$$\left.-\frac{1}{k_4+k_6}\{1-\exp(-(k_4+k_6)t)\}\right] \quad \text{eq. 13}$$

$$X(VI_t) = 1 - \{X(I_t)+X(II_t)+X(III_t)+X(IV_t)+X(V_t)\} \quad \text{eq. 14}$$

On the other hand, the mol fractions of I, III and V at time t can be obtained from HPLC data, using authentic compounds as standards. The mol fractions of II and of IV at time t can then be calculated from the data, using the specific ratios to the internal standards obtained from HPLC of sample solutions of pH 4 and pH 11. Further, the mol fraction of VI at time t can be calculated from equation 14. As shown in Fig. 10, the calculated values agree well with the observed values.

Fig. 11 shows the pH profile of each rate constant in Chart 6 at 40°C. The results of additional experiments and a discussion on the stability of CQ in aqueous solution based on the present results will be presented in subsequent papers.

It should be mentioned that reports by Poochikian and Cradock on the degradation of 2,5-diaziridinyl-3,6-bis(carbo-ethoxyamino)-1,4-benzoquinone in aqueous solution[16,17] became known to us after the present work had been completed. Their findings and interpretations are essentially consistent with these presented here.

Acknowledgement The authors are grateful to Dr. S. Akagi, director of these laboratories, and Dr. R. Okada for their kind encouragement and for permission to publish this article. Thanks are also due to Dr. W.I. Higuchi, College of Pharmacy, the University of Michigan, for many helpful suggestions during the preparation of this manuscript.

References and Notes

1) H. Nakao, M. Arakawa, T. Nakamura, and M. Fukushima, *Chem. Pharm. Bull.*, **20**, 1968 (1972).
2) H. Shindo, K. Nambu, E. Nakajima, and K. Kawai, *Yakugaku Zasshi*, **94**, 1393 (1974).
3) D.S. Tarbell and P. Noble, Jr., *J. Am. Chem. Soc.*, **72**, 2567 (1950).
4) V.B. Schatz and L.B. Clapp, *J. Am. Chem. Soc.*, **77**, 5113 (1955).
5) J.E. Earley, C.E. O'Rourke, L.B. Clapp, J.O. Edwards, and B.C. Lawes, *J. Am. Chem. Soc.*, **80**, 3458 (1958).
6) A. Anhalt and H. Berg, *J. Electroanal. Chem.*, **4**, 218 (1962).
7) W. Gauss and S. Peterson, *Angew. Chem.*, **67**, 226 (1955).
8) Milwaukee, Wisconsin 53233, U.S.A.
9) When Cl^- is present, the degradation of EB in acidic solutions is more coplex, because the ethylenimine ring splits to form an ethanolamino group or a chloroethylamino group (refs. 4 and 5).
10) In order to obtain complete dissolution of the residual substances after evaporation.
11) Hitachi gel 3010 (Hitachi Ltd., Tokyo, Japan).
12) LiChrosorb RP-18 (E. Merck, Darmstadt, Germany).
13) A.A. Frost and R.G. Pearson, "Kinetics and Mechanism," John Wiley and Sons, Inc., New York, 1953, p. 153.
14) C.K. Ingold, "Structure and Mechanism in Organic Chemistry," 2nd ed., Cornell University Press, Ithaca, 1969, pp. 1015—1023.
15) The aqueous solution containing peak IV component can be obtained from basic aqueous solution of EB at an early stage of the reaction by chloroform extraction.
16) G.K. Poochikian and J.C. Cradock, *J. Pharm. Sci.*, **70**, 159 (1981).
17) G.K. Poochikian and J.C. Cradock, *J. Pharm. Sci.*, **70**, 162 (1981).