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The Stability of Carboquone in Aqueous Solution. III.[1] Kinetics and Mechanisms of Degradation of Carboquone in Aqueous Solution

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The kinetics and mechanisms of the degradation of carboquone (CQ) in aqueous solution were studied and compared with those of 2,5-bis(1-aziridinyl)-3,6-dialkyl-1,4-benzoquinones [EBs: R=–H (EB); $-CH_3$ (MEB); $-CH(CH_3)_2$ (IPEB)] determined previously.

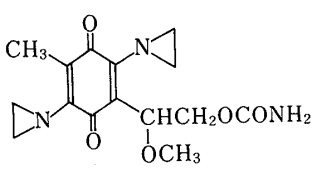
The degradation of CQ was essentially similar to those of EBs: it followed pseudo first-order kinetics (specific acid-base catalysis), and involved the hydrolytic cleavage of aziridine rings in acidic solutions, the substitution of aziridine rings by hydroxyl ion (radical) in basic solutions and a combination of these two mechanisms in solutions of neutral pH.

High performance liquid chromatography patterns of CQ solutions at acidic pH gave two peaks corresponding to two intermediate compounds with one aziridine ring and one hydroxyethylamino group (due to the asymmetrical structure of CQ). However, we could not identify each peak component, or estimate which aziridine ring (at the 2 or 5 position of CQ) was unreacted. This was also the case for the two intermediate compounds with one aziridine ring and one hydroxyl group observed in CQ solutions at basic pH.

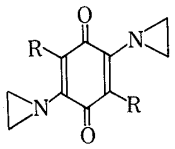
Kinetic studies of MEB and the two intermediate compounds obtained in acidic solutions by thin-layer chromatography separation made it possible to estimate the chemical structures of these four intermediate compounds.

Keywords——carboquone; antitumor agent; aziridinyl benzoquinone; hydroxyethylamino benzoquinone; hydroxy benzoquinone; degradation kinetics; degradation mechanisms; HPLC

Carboquone [CQ: 2,5-bis(1-aziridinyl)-3-(2-carbamoyloxy-1-methoxyethyl)-6-methyl-1,4-benzoquinone][2,3] is one of the (1-aziridinyl)benzoquinone type (Chart 1) alkylating agents with antitumor activity. In the previous two reports,[1,4] three kinds of 2,5-bis(1-aziridinyl)-3,6-dialkyl-1,4-benzoquinones (EBs: R=–H, $-CH_3$ and $-CH(CH_3)_2$, Chart 1) were chosen as model compounds of CQ, and the kinetics and mechanisms of the degradation of EBs in aqueous solution were studied. EBs were found to undergo changes of the two aziridine rings: sequential hydrolytic cleavage of them in acidic solutions, sequential substitution of them by hydroxyl ion (radical) in basic solutions, and a combination of these two mechanisms in solutions of neutral pH.



CQ



EB : R=−H
MEB : R=−CH_3
IPEB : R=−$CH(CH_3)_2$

Chart 1

CQ may be expected to be degraded by mechanisms similar to those for EBs with respect to the aziridine moiety, but more degradation products will be formed due to the asymmetrical structure of CQ. Furthermore, probable changes of the carbamoyloxymethoxyethyl moiety would make the degradation mechanisms of CQ more complicated. The present study deals with the degradation mechanisms of CQ, focussing mainly on the degradation of the aziridine moiety.

Experimental

Materials——CQ was obtained from a regular manufacturing batch (mp 203 °C). *Anal.* Calcd for $C_{15}H_{19}N_3O_5$: C, 56.07; H, 5.91; N, 13.08. Found: C, 56.17; H, 6.05; N, 13.19.

2,5-Dihydroxy-3,6-dialkyl-1,4-benzoquinones were obtained as previously reported.[1,4] Other chemicals used were of the highest grade commercially available.

Buffer Solutions for Kinetic Study——Acetate buffer (pH 4—5), phosphate buffer (pH 6—8), borate buffer (pH 9) and carbonate buffer (pH 10—12) were used. The concentration of each buffer solution was 0.01 M.

Procedure for Kinetic Study——i) CQ Stock Solution: CQ (500 mg) was dissolved in *N*,*N*-dimethylacetamide (DMA) and made up to 100 ml.

ii) Degradation Kinetics of CQ: Aliquots (1 ml each) of CQ stock solution were mixed with 49 ml of buffer solution equilibrated in a water bath thermostated at 20, 30, 40 or 50 °C ± 0.1 °C. At regular intervals, 5 ml samples was taken into 20 ml volumetric flasks. To each flask, 1 ml of 0.5 M triethanolamine–acetate buffer solution, pH 7 (to stop the reaction), and 2 ml of methyl salicylate solution (0.1% in methanol) as an internal standard were added, and the mixture was diluted to the mark with methanol. The solutions thus obtained were analyzed by means of high performance liquid chromatography (HPLC) (method I).

iii) Disappearance of CQ and Appearance of Degradation Products: After the mixing of 1 ml aliquots of CQ stock solution with 49 ml of buffer solution equilibrated in a water bath at 50 °C, the disappearance of CQ and the appearance of the degradation products were monitored by HPLC methods II and III. Samples for method II were prepared in the same way as for the study of the degradation kinetics of CQ described above (methyl salicylate solution was not added). Samples for method III were prepared as follows: 2 ml samples of the reaction mixture taken at regular intervals were each mixed with 1 ml of 0.5 M sodium bicarbonate aqueous solution to stop the reaction. The solutions thus obtained were loaded onto a column.

Separation of Degradation Products A and B by Means of Thin-Layer Cromatography (TLC)——Aliquots of CQ stock solution (4 ml each) were mixed with 46 ml of acetate buffer, pH 4, equilibrated in a water bath thermostated at 50 °C. After 15 min storage, the reaction mixture was poured into a separatory funnel containing 10 ml of chloroform and shaken vigorously. After standing for 10 min, the chloroform layer was collected and loaded onto silica gel TLC plates,[5] which were developed in chloroform–methanol (10 : 1). After development, the areas corresponding to *Rf* values of 0.35 (A) and 0.22 (B) were each collected, and extracted with 20% ethanol in chloroform. Each extract was evaporated to dryness and the residue was dissolved in DMA. The DMA solutions of A and B thus obtained were used to study their degradation kinetics (pH 4.1 and 10.9, 50 °C).

HPLC——Chromatography was performed on a Hitachi liquid chromatograph, model 635, equipped with a monitoring system at a wavelength of 325 nm. Column A (5 mm i.d. × 50 cm) of porous styrene–divinylbenzene copolymer[6] and column B (4 mm i.d. × 15 cm) of octadecylsilane chemically bonded to totally porous silica gel[7] were used. The chromatographic conditions are listed in Table I.

Results and Discussion

Degradation Kinetics of CQ

The degradation of CQ follows pseudo first-order kinetics over a wide range of pH. The pH-rate profile is shown in Fig. 1 together with those of EB, MEB and IPEB. The slopes in the acidic region and in the basic region were −1 and +1, respectively. This indicates that the degradation of CQ is subject to specific acid-base catalysis, as is the case for EB, MEB and IPEB.[1,4] Figure 1 also shows that CQ is less stable than MEB over the pH range studied, and this tendency is especially marked in the basic region. This phenomenon could be ascribed to the increase in the lability of the aziridine ring at the 2 position due to the presence of the carbamoyloxymethoxyethyl group at the 3 position of CQ.

Apparent activation energies obtained from Arrhenius plots at pH 4 and 11 are listed in

Table I. HPLC Conditions

Method	Column	Mobile phase	Flow rate (ml/min)	Temp. (°C)
I	A	5% 10 mM $NaHCO_3$/MeOH	2.0	40
II	A	40% 10 mM $NaHCO_3$/MeOH	1.5	40
III	B	5% MeOH/50 mM $NaHCO_3$	1.0	50

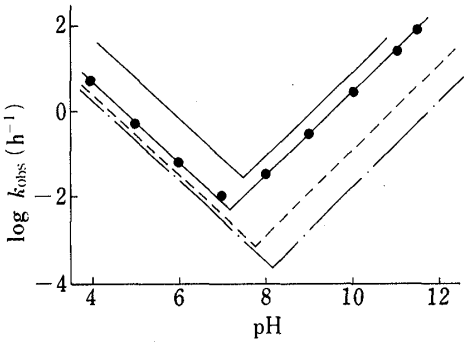


Fig. 1. $\log k_{obs}$-pH Profiles for the Degradation of CQ (●) at 50 °C

Profiles for EB (——), MEB (– – – –) and IPEB (— – —) were obtained previously.[1,4]

Table II. Apparent Activation Energies Obtained from Arrhenius Plots

Compound	Activation energy (kcal/mol)	
	pH 4	pH 11
CQ	18	23
EB	14	19
MEB	16	24
IPEB	17	23

Values for EB, MEB and IPEB were obtained previously.[1,4]

Table II together with those of EB, MEB and IPEB.[1,4] Again, similar relationships exist among the compounds.

Degradation Mechanism of CQ in Aqueous Solutions of pH 4—6

Figure 2(ia) shows a typical HPLC pattern of CQ solution stored at acidic pH; three peaks, *i.e.* A, B and C, are observed as degradation products during the initial two half-lives. At pH 4.1, 50 °C, where CQ is lost at a rate of 5.3 h^{-1}, peaks A and B increased gradually to attain maxima at around 12—13 min, but then decreased with time. On the other hand, peak C increased gradually after a short lag time. The ratio of the peak area of A to that of B almost remained constant (7 to 3) during the reaction.

Both A and B (obtained by TLC separation) showed similar UV spectra (λ_{max} = 345 nm in methanol), and were transformed to C in acidic aqueous solution at almost the same rate (4.1 h^{-1} at pH 4.1, 50 °C). In addition, the ultraviolet (UV) spectrum of A and its *Rf* value on TLC coincided with those of one of the degradation products of CQ in aqueous solution, whose structure was proposed to be I or II (Chart 2) on the basis of mass spectral data by Kawada[8]: it has one inert aziridine ring (at the 2 or the 5 position) and one hydroxyethylamino group derived from the other aziridine ring (at the 5 or the 2 position) by hydrolytic ring cleavage, but it could not be determined which aziridine moiety is hydrolyzed.

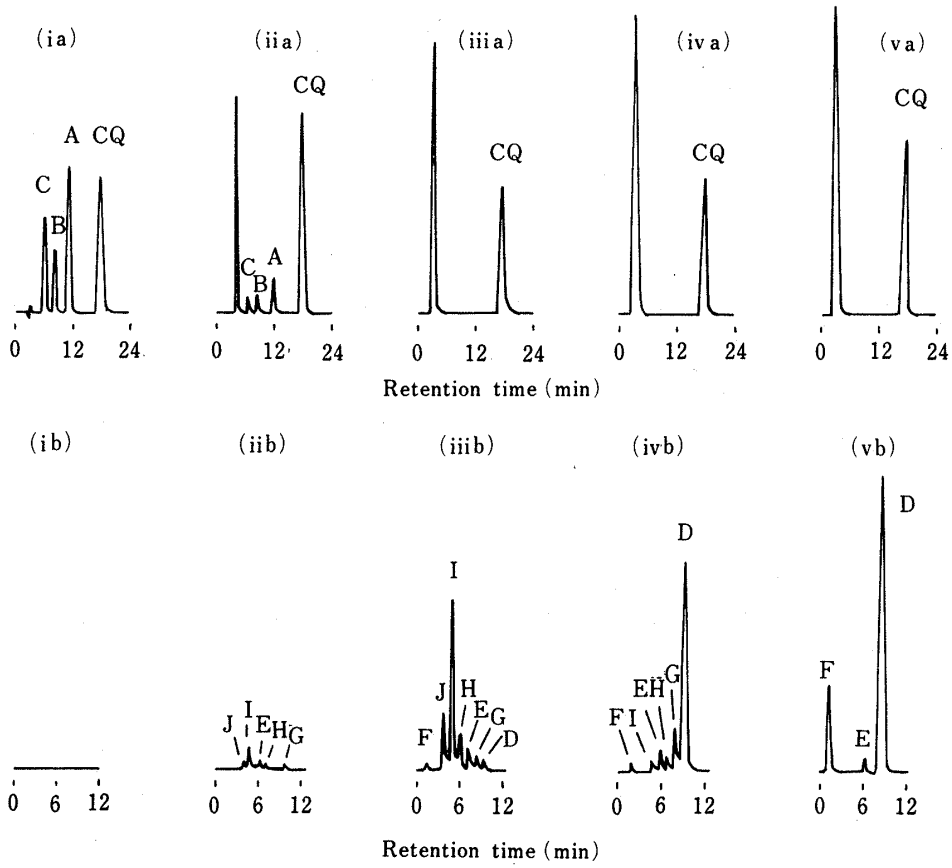
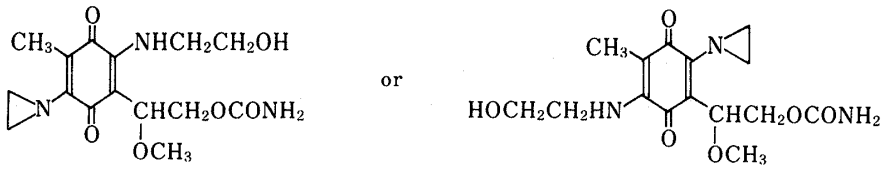


Fig. 2. HPLC Patterns of Aqueous Solutions of CQ Stored at 50 °C (a, by Method II; b, by Method III)

CQ remaining is shown in parentheses (%).
(ia) at pH 4.1 for 10 min (41%); (ib) at pH 4.1 for 60 min (0.5%); (iia, b) at pH 7.0 for 48 h (62%); (iiia, b) at pH 8.0 for 24 h (37%); (iva) at pH 9.0 for 2 h (40%); (ivb) at pH 9.0 for 4 h (16%); (va) at pH 10.9 for 2 min (50%); (vb) at pH 10.9 for 90 min (0%).



I II

Chart 2

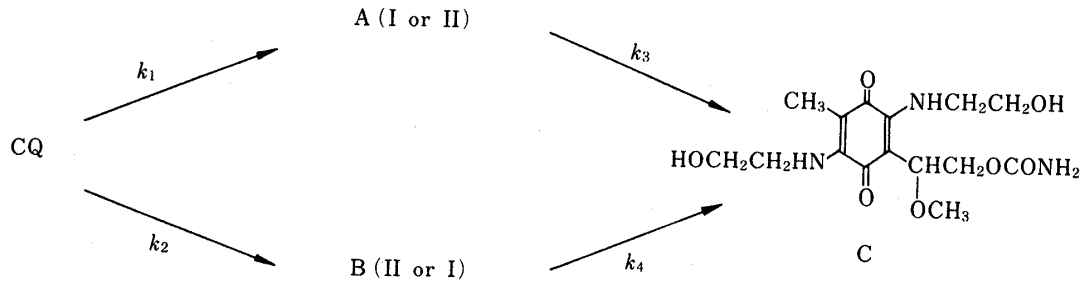


Chart 3

These data and the results for EB,[4] MEB and IPEB[1] indicate that A and B are intermediates in the formation of C, and the degradation mechanism of CQ in acidic aqueous solution is proposed to be as shown in Chart 3: parallel sequential hydrolytic cleavage of the

two aziridine rings.

When CQ is degraded according to Chart 3, the mol fractions of CQ, A, B and C at time t can be calculated from Eqs. 1, 2, 3 and 4, respectively.[9]

$$X(\mathrm{CQ}_t)=\exp\left(-(k_1+k_2)t\right) \tag{1}$$

$$X(\mathrm{A}_t)=\frac{k_1}{k_3-(k_1+k_2)}\{\exp(-(k_1+k_2)t)-\exp(-k_3t)\} \tag{2}$$

$$X(\mathrm{B}_t)=\frac{k_2}{k_4-(k_1+k_2)}\{\exp(-(k_1+k_2)t)-\exp(-k_4t)\} \tag{3}$$

$$X(\mathrm{C}_t)=1-\{X(\mathrm{CQ}_t)+X(\mathrm{A}_t)+X(\mathrm{B}_t)\} \tag{4}$$

Experimentally, k_1+k_2 ($=k_{obs}$), k_3 and k_4 can readily be obtained from the degradation kinetics of CQ, A and B, respectively. Then, k_1 and k_2 can be obtained by proportional allotment of k_{obs} on the assumption that the k_1/k_2 ratio is equal to the ratio of the peak area of A to that of B during the reaction, which is reasonable on the basis of the similarities of chemical structures. The time courses of peak heights corresponding to CQ, A, B and C showed patterns quite similar to the calculated ones. The ratio of peak height at around the first half-life (10 min in the case of pH 4.1, 50 °C) to the calculated value at that time was used as the basis for fitting the curves.

As shown in Fig. 3, the calculated values agree well with the observed values up to two half-lives, assuming $k_1=3.7\,\mathrm{h}^{-1}$, $k_2=1.6\,\mathrm{h}^{-1}$ and $k_3=k_4=4.1\,\mathrm{h}^{-1}$, respectively. The k_2 value is very close to the rate constant of MEB ($1.8\,\mathrm{h}^{-1}$) under these conditions.[1] The deviation between calculated and experimental values for C after 20 min could be ascribed to further degradation (probably a change of the carbamoyloxymethoxyethyl moiety). This is supported by the fact that four additional peaks were observed after 30 min by HPLC method II.

Therefore, the degradation mechanism of CQ in acidic aqueous solution is concluded to be as shown in Chart 3.

Estimation of the Chemical Structures of A and B

A and B were degraded at the same rate in acidic aqueous solution, as mentioned above, but A was much more stable than B in basic aqueous solution. On the basis of this phenomenon, we can speculate as to which aziridine ring of CQ is left unreacted. At pH 10.9, 50 °C, the rate constants for A and B were 1.7 and $20\,\mathrm{h}^{-1}$, respectively, with the former value being very close to the rate constant of MEB under the same conditions, $1.3\,\mathrm{h}^{-1}$.[1] This

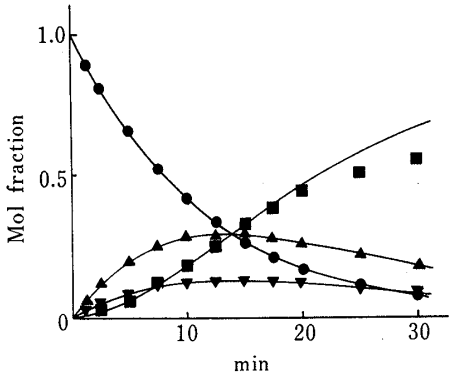


Fig. 3. Time Courses of CQ (●), A (▲), B (▼) and C (■) during CQ Degradation at pH 4.1, 50 °C

The lines are calculated values based on Chart 3.

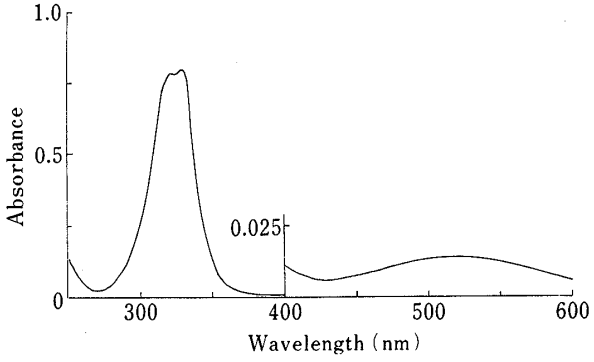


Fig. 4. UV-Visible Spectrum of F

After storage of CQ (200 μg/ml; 6.23×10^{-4} M) in 0.1 N NaOH solution at 50 °C for 100 min, the reaction mixture was diluted 10-fold with water, and the UV-visible spectrum of the solution thus obtained was measured.

strongly suggests that A retains the aziridine ring which is more stable in basic aqueous solution, *i.e.* the one at the 5-position. Therefore, the structures of A and B are concluded to be I and II, respectively.

Degradation Mechanism of CQ in Aqueous Solutions of pH 10—12

A typical HPLC pattern (method II) of CQ solution stored at basic pH is shown in Fig. 2(va) and is quite different from that at acidic pH (Fig. 2(ia)): peaks A, B and C were not observed and another peak(s) appeared at the solvent front. The last was resolved into three peaks (D, E and F) by method III (Fig. 2(vb)). At pH 10.9, 50 °C, the rapid loss of CQ (at a rate of 20.5 h^{-1}), coincided with rapid increases of peaks D and E during the initial stage of the reaction, but peaks D and E later decreased gradually, while peak F gradually increased after a short lag time. The ratio of peak area of D to that of E remained at 93 to 7 during the initial 30 min and increased later (95 to 5 at 120 min and 97 to 3 at 300 min). The rate constants for D and E were obtained as 0.086 and 0.26 h^{-1} after 120 min.

When CQ was stored in 0.1 N NaOH solution for 100 min at 50 °C, the solution contained only F. The UV-visible spectrum of the solution was quite similar to those of 2,5-dihydroxy-3,6-dialkyl-1,4-benzoquinone compounds (Fig. 4 and Table III). This strongly suggests that F is 2,5-dihydroxy-3-(2-carbamoyloxy-1-methoxyethyl)-6-methyl-1,4-benzoquinone.

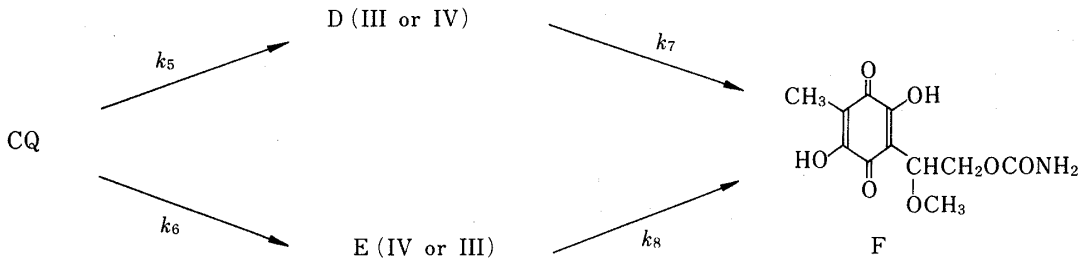
These data and the results for EB,[4] MEB and IPEB[1] indicate that D and E are intermediates in the formation of F, and the degradation mechanism in basic aqueous solution is proposed to be as shown in Chart 4: parallel sequential substitution of the two aziridine rings by hydroxyl ion (radical).

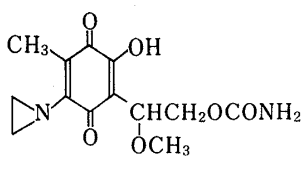
When CQ is degraded according to Chart 4, the mol fractions of CQ, D, E and F at time t

Table III. Spectral Data for F and Dihydroxy-benzoquinones in Basic Aqueous Solution

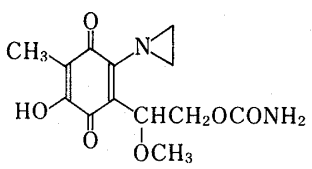
Compound	λ_{max} nm, (log ε)
F	322, 329 (4.40); 521 (2.37)
2,5-Dihydroxy-1,4-benzoquinone	316, 324 (4.28); 497 (2.30)
2,5-Dihydroxy-3,6-dimethyl-1,4-benzoquinone	327,[a] 334 (4.48); 550 (2.35)
2,5-Dihydroxy-3,6-diisopropyl-1,4-benzoquinone	327,[a] 333 (4.41); 550 (2.36)

a) Shoulder.





III



IV

Chart 4

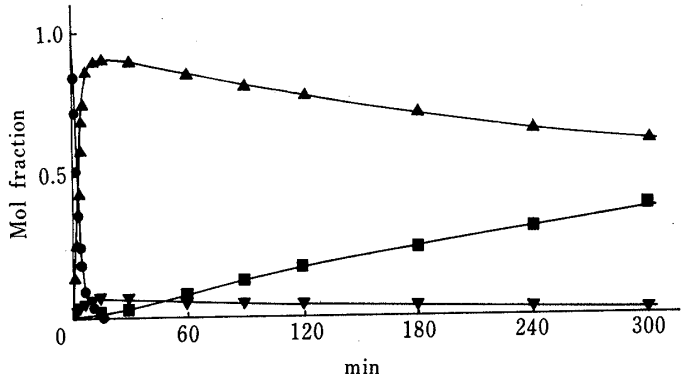


Fig. 5. Time Courses of CQ (●), D (▲), E (▼) and F (■) during CQ Degradation at pH 10.9, 50 °C

The lines are calculated values based on Chart 4.

can be calculated from Eq. 1—4 as in the case of CQ in acidic aqueous solution.

Experimentally, k_5+k_6 ($=k_{obs}$) can be readily obtained from the degradation kinetics of CQ. Also, k_7 and k_8 can be obtained from the rates of decrease of D and E, respectively, after complete disappearance of CQ in the basic solution. Just as in the case of k_1 adn k_2 above, k_5 and k_6 could be obtained by proportional allotment of k_{obs} on the assumption that the k_5/k_6 ratio is equal to the ratio of the peak area of D to that of E during the initial part of the reaction (up to around the time of their maximal peak areas), which is again reasonable on the basis of the similarities of their chemical structures. The time courses of peak heights corresponding to CQ, D, E and F showed patterns quite similar to the calculated ones. The ratio of peak height at an appropriate time (2 min for CQ, and 60 min for D, E and F at pH 10.9, 50 °C) to the calculated value at that time was used as the basis for fitting the curves, just as in the case of CQ in acidic aqueous solution.

As shown in Fig. 5, the calculated values agreed well with the observed values, assuming $k_5=19\,h^{-1}$, $k_6=1.5\,h^{-1}$, $k_7=0.086\,h^{-1}$ and $k_8=0.26\,h^{-1}$.

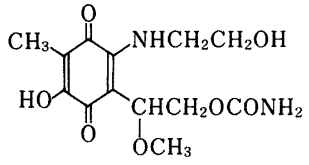
Therefore, the degradation mechanism of CQ in basic aqueous solution is concluded to be as shown in Chart 4.

Estimation of the Chemical Structures of D and E

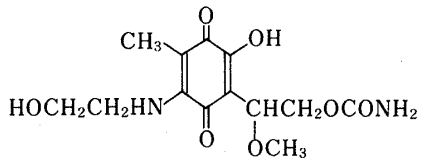
CQ is less stable than MEB by a factor of 16 in basic aqueous solution (Fig. 1) and this could be ascribed to the enhanced lability of the aziridine ring at the 2 position due to the presence of the carbamoyloxymethoxyethyl group at the 3 position, as mentioned above. This suggests that D has a 2-hydroxy-5-aziridinyl structure and E has a 2-aziridinyl-5-hydroxy structure. This speculation is supported strongly by the stability difference between A (2-hydroxyethylamino-5-aziridinyl structure) and B (2-aziridinyl-5-hydroxyethylamino structure) in basic aqueous solution by a factor of about 12 as mentioned above. Therefore, the structures of D and E are concluded to be III and IV, respectively.

Degradation Mechanism of CQ in Aqueous Solutions of Intermediate pH

In the intermediate pH region, CQ is expected to be degraded by a combination of the two mechanisms: hydrolytic cleavage observed in the pH 4—6 range, and substitution of the aziridine ring by hydroxyl ion (radical) observed in the pH 10—12 range. In addition to A, B, C, D, E and F, therefore, V and VI (Chart 5) are expected to be formed from either A (by substitution of the aziridine ring at the 5 position by hydroxyl ion (radical)) or E (by hydrolytic cleavage of the aziridine ring at the 2 position), and from either B (by substitution of the aziridine ring at the 2 position by hydroxyl ion (radical)) or D (by hydrolytic cleavage of the aziridine ring at the 5 position), respectively, as degradation compounds.



V



VI

Chart 5

At pH 7, peaks A, B and C were dominant, although peaks E, G, H, I and J were observed as degradation products (Fig. 2(iia, b)), while at pH 8 and 9, peaks D, E, F, G, H, I and J were seen (Fig. 2(iiia, b) and (iva, b)). A solution consisting predominantly of D and E was obtained from CQ aqueous solution stored at pH 10.9, 50 °C for 10 min by chloroform extraction. On acidification of this solution with acetic acid, peaks G and H appeared followed by peaks I and J. Also, adjustment of the pH values of the solution to 8 and 9 gave HPLC patterns similar to Fig. 2(iiib) and(ivb), respectively, after storage for several hours at 50 °C. On the other hand, A and B were observed to be degraded to G and H, respectively, in basic aqueous solution. Consequently, the structures of G and H can be estimated at VI and V, respectively, and I and J may be their further degradation products (on the basis of the probable changes of the carbamoyloxymethoxyethyl moiety).

Therefore, CQ is concluded to be degraded by a combination of hydrolysis and substitution of the aziridine rings, together with further degradation of the carbamoyloxymethoxyethyl moiety.

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