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Stability of (+)-Cyanidanol-3 in Aqueous Solution

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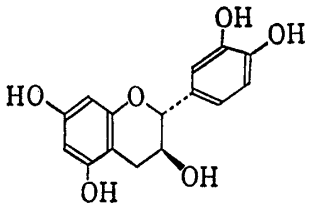
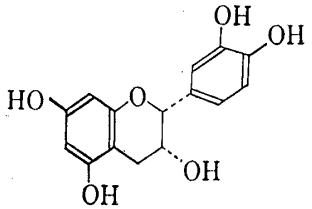
The stability of (+)-cyanidanol-3 in aqueous solution was investigated. Two degradation products were found at pH 1.4 by TLC. These were assumed to be epicatechin and the dimer. In the basic pH region, the main product was found to be epicatechin. It was most stable at about pH 3 and was labile in strongly acidic and basic solutions. The effect of oxygen in the head space of the container was remarkable, especially in basic solution. In strongly acidic solution, the degradation of (+)-cyanidanol-3 appeared to occur by simultaneous apparent 1st-order (epimerization) and 2nd-order (dimerization) reactions. The rate constants were calculated by a new method. In the neutral and basic pH regions, the reverse reaction between (+)-cyanidanol-3 and epicatechin took place and the rate constants were obtained.

Keywords——(+)-cyanidanol-3; stability; kinetic; epicatechin; hepatoprotector; epimerization; dimerization

(+)-Cyanidanol-3, KB-53, is one of the flavans obtained from wood and leaves of *Uncaria gambir*, and its hepatoprotective activity has been described in the recent literature.[1] Since this hepatoprotector will be manufactured in liquid dosage forms, for example, for intravenous injection, its solution stability profile should be well characterized and understood. The formation of polymers during the autoxidation of KB-53 in aqueous solution has been reported by Hathway and Seakins.[2] They revealed that phlobatannins which were obtained from the leaves of *Uncaria gambir* had similar analytical properties to the polymers obtained by KB-53 autoxidation. On the other hand, Freudenberg *et al.*[3] reported the formation of KB-53 dimer by acid condensation, and its structure was recently determined by Byung-Zun Ahn *et al.*,[4] by mass spectrometry. Further, it has been shown by Courbat *et al.*[5] and Sears *et al.*[6] that KB-53 rearranged on heating in basic solution to catechinic acid. However, no other quantitative study on the stability of KB-53 in aqueous solution has been reported. In this report, we describe its degradation products, the effect of pH and oxygen on its stability and the kinetics of its degradation in basic and acidic solutions.



(+)-cyanidanol-3



epicatechin

Experimental

Materials——KB-53 was obtained from Zyma S. A. upon recrystallization three times from water followed by drying over P_2O_5. (±)-Epicatechin was obtained from Zyma S. A. and other chemicals were of reagent grade quality. Deionized, then distilled water was used in all experiments.

Kinetic Measurement——KB-53 ranging from 6.8×10^{-4} M to 8.6×10^{-3} M was dissolved in buffer solutions and flushed with nitrogen for 30 min at 100 ml/min. Buffer compositions are shown in Table I. The ionic strength of the buffer solution was adjusted to 0.15 with sodium chloride. The pH at the temperature of

the kinetic runs was measured with a digital pH meter (Hitachi digital pH meter, model F-7DE). Eleven milliliters of the solution was then placed in a 10 ml glass ampule, and flushed again with nitrogen for 10 min at 60 ml/min. The ampule was sealed and immersed in a controlled temperature water bath at 40—50± 0.2°C. Ampules were withdrawn at appropriate intervals and cooled to room temperature.

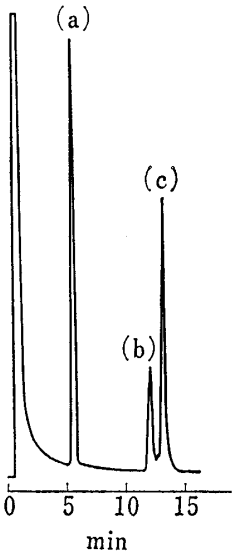


Fig. 1. Gas Chromatogram of Trimethylsilylated Internal Standard (a), Epicatechin (b) and KB-53 (c)

Assay Method——The concentrations of KB-53 and epicatechin were assayed by the following GLC method. To 5 ml of the solution in the ampule was added 10 ml of ethyl acetate. In the case of the kinetic run at pH 9.6, 1 ml of 1 N HCl and 10 ml of ethyl acetate were added to 5 ml of the solution. In the case of the run at pH 10.7, 1.5 ml of 1 N HCl and 10 ml of ethyl acetate were added to 5 ml of the solution. The mixture was then shaken and centrifuged. Two milliliters of the organic layer was evaporated to dryness and the residue was dissolved in 1 ml of internal standard solution containing 1 mg of dehydroepiandrosterone and 1 ml of pyridine. To this solution, 250 μl of bis-(trimethylsilyl)-trifluoroacetamide was added. The reaction mixture was allowed to stand for 30 min and then 1 μl was injected into a gas–liquid chromatograph. GLC was performed on a 2 m × 3 mm i.d. glass column packed with 2% silicone UC W-98 chromosorb W. The injection port, column and detector temperatures were maintained at 310°C, 240°C and 310°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 50 ml/min. A representative gas-chromatogram of trimethylsilylated KB-53, epicatechin and internal standard is shown in Fig. 1. Quantitation was based on peak-height ratio comparison with linear standard plots.

Results and Discussion

Identification of Main Degradation Product

To determine the mechanism of degradation, the main degradation product was isolated and identified. Degraded solutions at pH 1.4 and 9.6 (40 h and 3 h, respectively, at 50 °C) were subjected to TLC on cellulose plates (Cellulose F, Merck) using H_2O–dioxane–AcOH (10: 1: 1) as the developing solvent, then the products were detected by means of 10% Na_2CO_3 aq. and 0.5% Echtblausalz B (Merck) aq. spray. The degradation products at pH 1.4 were nicely separated from KB-53; the *Rf* values for KB-53 and the two degradation products were 0.65, 0.50 and 0.73, respectively. The mobility of the spot of *Rf* 0.73 was identical with that of the dimer of KB-53 which was prepared according to the method reported by Byung-Zun Ahn *et al.*,[4] although further isolation and identification could not be carried out because of the low yield. At pH 9.6, the spot of intact KB-53 and two spots of degradation products (main product *Rf* 0.50 and minor product *Rf* 0.71) were detected. The *Rf* value of the main degradation product (*Rf* 0.50), which was also detected in the case of pH 1.4, was identical with that of an authentic sample of epicatechin. In addition, column chromatography for identification was carried out. Eight hundred milligrams of KB-53 was dissolved in 400 ml of pH 9.6 buffer solution and heated at 60 °C under a stream of nitrogen for 3 h. The degraded solution was extracted twice with 400 ml of ethyl acetate. The extracted solution was concentrated under reduced pressure and the residue was dissolved in 10 ml of H_2O–EtOH (1: 1) mixture. This extract was subjected to column chromatography for separation of the main product. Two hundred grams of cellulose CF-11 (Whatman) was used as the support and H_2O–EtOH (10: 1) mixture as the eluent. The eluate containing the spot (*Rf* 0.50) was concentrated under reduced pressure to provide crude crystals (180 mg). Five milliliters of acetic anhydride and 0.5 ml of pyridine were added to this residue and the mixture was allowed to stand overnight. Next, 40 ml of water was added and the whole was extracted twice with 30 ml of chloroform. The chloroform layer was washed with water, dried over $MgSO_4$ and

concentrated under reduced pressure. Preparative thin-layer chromatography was used for further purification of the acetylated degradation product: Kieselgel 60 F_{254} (Merck), and the developing solvent system was ethyl acetate–hexane (2: 3). Recrystallization from methanol afforded white plates (110 mg). mp 149—150 °C. NMR ($CDCl_3$): 1.99 (3H, s, 3-acetyl CH_3), 2.43 (12H, s, 5,7,3′,4′-acetyl CH_3), 2.7—3.0 (2H, m, 4-CH_2), 4.9—5.2 (1H, m, 2-CH), 5.2—5.5 (1H, m, 3-CH), 6.4—6.7 (2H, m, 8-arom. and 4-arom.), 7.0—7.4 (3H, m, 2′,5′,6′-arom.). The NMR spectrum of the acetylated degradation product was identical with that of the pentaacetate of epicatechin which was prepared independently from an authentic sample of epicatechin in acetic anhydride–pyridine mixture. In addition, the retention time of the main degradation product in gas–liquid chromatography after trimethylsilylation was identical with that of an authentic sample of epicatechin similarly treated. With respect to the minor product (*Rf* 0.71), further analysis was not carried out in the present work.

Effect of pH

The logarithm of the amount of KB-53 remaining in aqueous solution not flushed with nitrogen was plotted as a function of time. Fig. 2 shows that log concentration *versus* time plots were not linear over the pH range of 1—11, so that KB-53 was assumed to decompose according to a complex reaction. The 25% loss time ($T_{3/4}$) of KB-53 was measured graphically from Fig. 2 and is shown in Table I. As shown in Table I, 6.9×10^{-3} M KB-53 in aqueous solution was most stable at about pH 3 and was labile in basic and strongly acidic solutions.

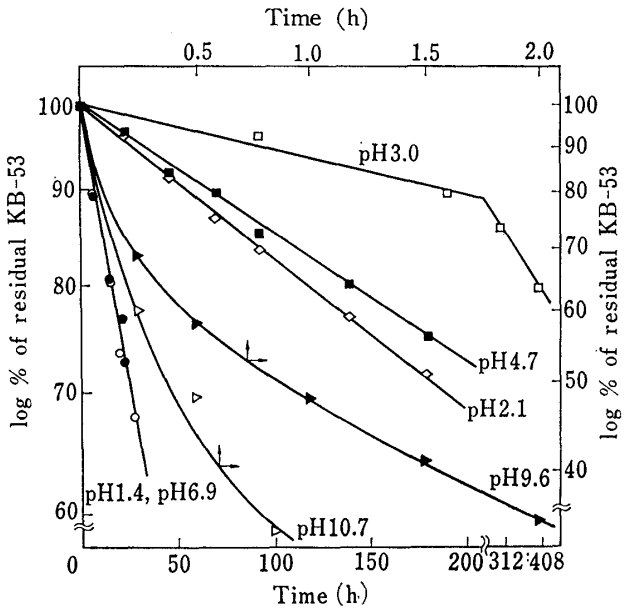


Fig. 2. Decomposition of 6.9×10^{-3} M KB-53 in Aqueous Solution not flushed with Nitrogen at 50°C

Table I. 25% Loss Time of KB-53 ($T_{3/4}$) in Aqueous Solution[a] at 50°C

pH	Buffer composition	$T_{3/4}$ (h)
1.48 (1.44)[b]	0.077 M HCl, 0.060 M KCl, 0.013 M NaCl	21
2.13 (2.07)[b]	0.015 M HCl, 0.005 M glycine, 0.130 M NaCl	1.5×10^{2}
3.13 (3.04)[b]	0.004 M HCl, 0.016 M Glycine, 0.130 M NaCl	5.2×10^{2}
4.81 (4.71)[b]	0.050 M CH_3COOH, 0.050 M CH_3COONa, 0.050 M NaCl	1.9×10^{2}
6.93 (6.86)[b]	0.006 M Na_2HPO_4, 0.003 M NaH_2PO_4, 0.130 M NaCl	23
9.56 (9.56)[b]	0.060 M $NaHCO_3$, 0.030 M Na_2CO_3	6.2×10^{-1}
10.74 (10.74)[b]	0.005 M $NaHCO_3$, 0.045 M Na_2CO_3, 0.010 M NaCl	2.7×10^{-1}

a) 6.9×10^{-3} M solution not flushed with nitrogen.
b) pH value of final solution.

Effect of Oxygen

KB-53 is known to be easily oxidized by air and its oxidation mechanism was reported by Hathway *et al.*[2] It was found by a preliminary experiment that the aqueous solution not flushed with nitrogen produced a yellow to wine-red color after heating, especially in a basic solution. On the other hand, the solution flushed with nitrogen remained clear after heating at all pH regions. As shown in Table II, the influence of oxygen in the ampule on the stability

of KB-53 was more marked with increasing pH value. The KB-53 aqueous solution not flushed with nitrogen at pH 10.7 produced a wine-red color on heating at 40 °C for 16 h, and the solution exhibited absorption maxima at 425 nm and 525 nm. The absorption maxima of intact KB-53 and epicatechin were both at 280 nm. This absorption spectrum resembled that of polymer obtained by autoxidation of KB-53 reported by Hathway *et al.*[2d]

Kinetic Studies in Acidic Solution

The stability of KB-53 in acidic aqueous solution at pH 1.4 and 50 °C was measured in two cases, *i.e.*, at initial concentrations of 8.3×10^{-3} M and 8.3×10^{-4} M, as shown in Fig. 3. As mentioned above, it was found that KB-53 mainly decomposed to the dimer of KB-53 and epicatechin. Hence, it can be assumed that the degradation involved a simultaneous apparent first-order reaction (KB-53→epicatechin) and apparent second-order reaction (KB-53→dimer of KB-53) as expressed by equation (1),

$$-\mathrm{d[KB\text{-}53]/dt} = k_1[\mathrm{KB\text{-}53}] + k_2[\mathrm{KB\text{-}53}]^2 \qquad (1)$$

where [KB-53] is the molar concentration of KB-53 at time t, and k_1 and k_2 are the rate constants of epimerization and dimerization, respectively. The rate constants were obtained from the results in Fig. 3 by the following method.

Integration of equation (1) gives equation (2),

$$\frac{1}{k_1/k_2}\ln\left[\frac{[\mathrm{KB\text{-}53}]_0}{(k_1/k_2)+[\mathrm{KB\text{-}53}]_0}\cdot\frac{(k_1/k_2)+[\mathrm{KB\text{-}53}]}{[\mathrm{KB\text{-}53}]}\right] = k_2t \qquad (2)$$

where $[\mathrm{KB\text{-}53}]_0$ is the initial concentration of KB-53. If the remaining ratio, a, of KB-53 is defined by

$$a = [\mathrm{KB\text{-}53}]/[\mathrm{KB\text{-}53}]_0$$

equation (2) becomes

$$\frac{1}{k_1/k_2}\ln\left[\frac{1}{k_1/(k_2[\mathrm{KB\text{-}53}]_0)+1}\cdot\frac{k_1/(k_2[\mathrm{KB\text{-}53}]_0)+a}{a}\right] = k_2t \qquad (3)$$

On the other hand, equation (3′) is obtained similarly in the case of the initial concentration of $[\mathrm{KB\text{-}53}]_0'$,

$$\frac{1}{k_1/k_2}\ln\left[\frac{1}{k_1/(k_2[\mathrm{KB\text{-}53}]_0')+1}\cdot\frac{k_1/(k_2[\mathrm{KB\text{-}53}]_0')+a'}{a'}\right] = k_2t \qquad (3')$$

where a' is the remaining ratio of KB-53 at time t in the solution of initial concentration $[\mathrm{KB\text{-}53}]_0'$. Combining equation (3) and (3′) yields

Table II. $T_{3/4}$ of KB-53 Aqueous Solution (6.9×10^{-3} M) flushed or not flushed with Nitrogen at 50°C

pH	Flushing with N_2	$T_{3/4}$ (h)
1.4	+	22
	−	21
4.5	+	1.1×10^2
	−	7.0×10
7.0	+	24
	−	18
9.6	+	9.9×10^{-1}
	−	6.2×10^{-1}
10.7	+	5.2×10^{-1}
	−	2.7×10^{-1}

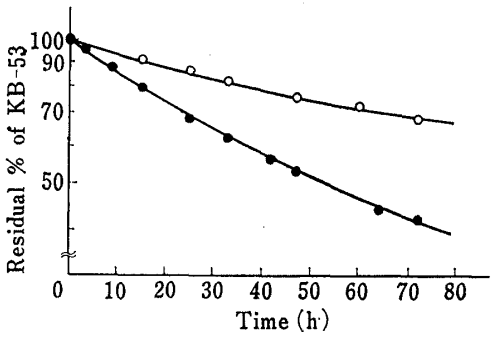


Fig. 3. Effect of Initial Concentration of KB-53 at pH 1.4 and 50°C

Initial concentration: ●, 8.3×10^{-3} M; ○, 8.3×10^{-4} M.
Key: ●, ○ experimental values, —— theoretical curve obtained from Eq. (2).

$$\frac{1}{K+1}\cdot\frac{K+a}{a}=\frac{1}{K'+1}\cdot\frac{K'+a'}{a'} \quad (4)$$

where K is $k_1/(k_2[\text{KB-53}]_0)$ and K' is $k_1/(k_2[\text{KB-53}]_0')$.

In our experiment, K' is equal to $10K$ because $[\text{KB-53}]_0$ is 8.3×10^{-3} M and $[\text{KB-53}]_0'$ is 8.3×10^{-4} M. Consequently equation (4) becomes equation (5).

$$10K(a'-a)=10a-a'-9aa' \quad (5)$$

Therefore K can be obtained from the slope of the plots of $(10a-a'-9aa')$ against $(a'-a)$, as shown in Fig. 4, where a and a' are the remaining ratios of intact KB-53 at the same time t in the solutions of initial concentration 8.3×10^{-3} M and 8.3×10^{-4} M, respectively. From the slope illustrated in Fig. 4 (correlation coefficient, 0.994), K was calculated to be 0.37. Furthermore, equation (3) becomes equation (6),

$$\ln\left(\frac{1}{K+1}\cdot\frac{K+a}{a}\right)=k_1t \quad (6)$$

where K is 0.37 as obtained above, and the plots of $\ln[(K+a)/a(K+1)]$ against time were found to be linear as shown in Fig. 5. From the slope illustrated in Fig. 5 (correlation coefficient, 0.999), k_1 was calculated to be 4.4×10^{-3} (h^{-1}). Substituting these values for K and k_1 into $K=k_1/(k_2[\text{KB-53}]_0)$ yielded a value of 1.4 (h^{-1} M^{-1}) for k_2. The experimental values in the case of both initial concentrations closely fitted the theoretical curves of equation (2) with $k_1=4.4\times10^{-3}$ (h^{-1}) and $k_2=1.4$ (h^{-1} M^{-1}), as represented by full lines in Fig. 3.

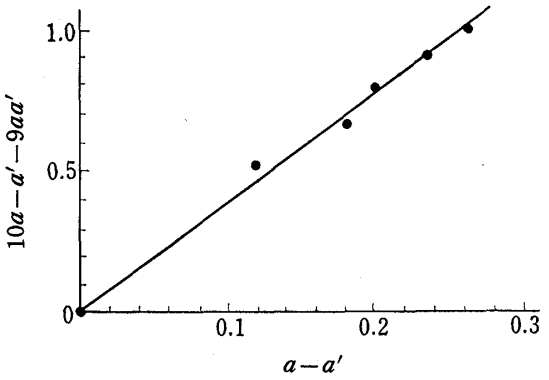


Fig. 4. Plot of Equation (5) at pH 1.4 and 50°C

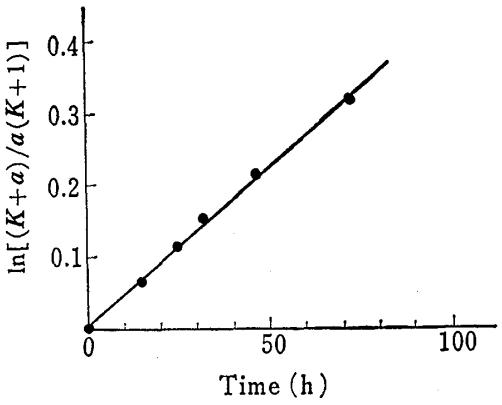


Fig. 5. Plot of Equation (6) at pH 1.4 and 50°C
$[\text{KB-53}]_0=8.3\times10^{-3}$ M

Kinetic Studies in Neutral and Basic Solutions

Epicatechin was the main degradation product in basic aqueous solution as mentioned before; however, the degradation of KB-53 did not follow an apparent first-order process in the alkaline pH region (Fig. 2). The disappearance of KB-53 and appearance of epicatechin were measured at pH 10.5 and 40 °C, and are shown in Fig. 6. Further, it was found that epicatechin decomposed to KB-53 at this pH (Fig. 7). Consequently, a reversible reaction between KB-53 and epicatechin was supposed to take place in basic solution. Furthermore, since the sum of KB-53 and epicatechin decreased with time, as represented by the dotted curve in Fig. 6, KB-53 also appears to decompose to other products than epicatechin. The same tendency as in Figs. 6 and 7 was observed at pH 9.6 and pH 6.9, so the following scheme of degradation of KB-53 in neutral and basic solutions was assumed, where each rate constant is a first-order rate constant (Chart 1).

These first-order rate constants were obtained by the method reported by Yamana *et al.*[7] Since the initial concentration of epicatechin is zero in Chart 1, the concentrations of KB-53 and

$$\text{other products} \xleftarrow{k_4} \text{KB-53} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{epicatechin} \xrightarrow{k_3} \text{other products}$$

Chart 1

epicatechin at time t can be expressed by equations (7) and (8), respectively.

$$[\text{KB-53}] = \frac{(k_1+k_4+m_2)[\text{KB-53}]_0}{m_2-m_1} e^{m_1 t} - \frac{(k_1+k_4+m_1)[\text{KB-53}]_0}{m_2-m_1} e^{m_2 t} \quad (7)$$

$$[\text{epicatechin}] = \frac{k_1[\text{KB-53}]_0}{m_2-m_1} (e^{m_1 t} - e^{m_2 t}) \quad (8)$$

where

$$m_1 = (1/2)\{-(k_1+k_2+k_3+k_4)-[(k_1+k_2+k_3+k_4)^2-4(k_2k_4+k_1k_3+k_3k_4)]^{1/2}\}$$

$$m_2 = (1/2)\{-(k_1+k_2+k_3+k_4)+[(k_1+k_2+k_3+k_4)^2-4(k_2k_4+k_1k_3+k_3k_4)]^{1/2}\}$$

As illustrated in Fig. 6, the ratio of [KB-53] to $[\text{KB-53}]_0$ and the ratio of [epicatechin] to $[\text{KB-53}]_0$ were plotted against time on a semilogarithmic scale. The slope of the plot relating to KB-53 was identical with that relating to epicatechin after approximately 2 h. The value of m_2 of equation (7) was measured graphically from this slope, then the value of $-(k_1+k_4+m_1)/(m_2-m_1)$ was measured graphically from the intercept of the straight line obtained by extrapolating the plot relating to KB-53 (arrow). In a similar manner, $k_1/(m_2-m_1)$ was measured graphically from the intercept of the straight line obtained by extrapolating the plot relating to epicatechin. The value of m_1 was measured graphically from the slope of the plot of $\ln\{-(k_1+k_4+m_1)e^{m_1 t}/(m_2-m_1)-[\text{KB-53}]/[\text{KB-53}]_0\}$ against time. Substituting these values for k_1, k_4, m_1 and m_2 into the following equations yielded the values of k_2 and k_3.

$$k_2 = -(m_1+m_2+k_1+k_3+k_4)$$

$$k_3 = (1/k_1)[m_1m_2+k_4(m_1+m_2+k_1+k_4)]$$

Table III shows the first-order rate constants (k_1—k_4) in Chart 1 measured from Figs. 6 and 7. Furthermore, Table III includes for comparison the rate constants at pH 9.6 and 6.9 measured in a similar manner at 40°C and 50°C, respectively. It was observed that the rate constant of epimerization of KB-53, that is k_1, increased as the pH increased in basic solution. On comparing the values of k_1 at pH 9.6 and pH 10.5 with that at pH 6.9, it appears that k_1 varied remarkably at around the pK_a of KB-53 (pK_{a_1}=8.97[8]) and k_2/k_1 was approximately constant above the pK_a. It is assumed that the rate constant of the epimerization of KB-53 is dependent on the ease of dissociation of the phenolic hydroxy function of the catechol moiety. The rate constants, k_3 and k_4, obtained from Fig. 6 were different from those obtained from Fig. 7, especially at pH 10.7. For this reason, it is considered that k_3 and k_4 have large experimental errors, because these constants were secondarily obtained after graphical calculation of k_1. On the other hand, it may be ascribed to a difference in reactivity between KB-53 and epicate-

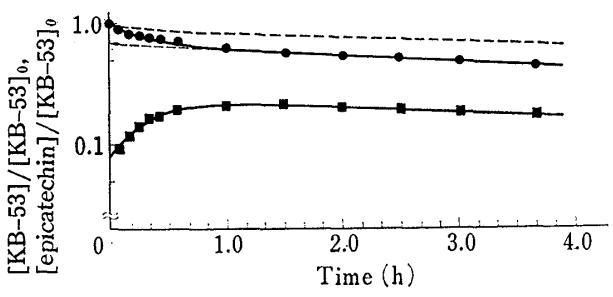


Fig. 6. Disappearance of KB-53 and Appearance of Epicatechin at pH 10.7 and 40°C

—●—, KB-53; —■—, epicatechin.
Initial concentration of KB-53: 6.9×10^{-3} M.

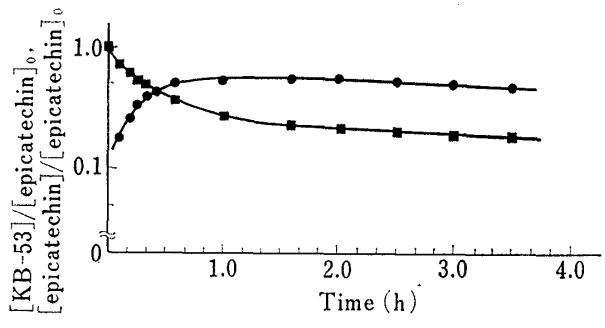


Fig. 7. Disappearance of Epicatechin and Appearance of KB-53 at pH 10.7 and 40°C

—○—, KB-53; —■—, epicatechin.
Initial concentration of epicatechin; 6.9×10^{-3} M.

TABLE III. First-Order Rate Constants of Chart 1

pH (Temp.)	From	(h^{-1}) k_1	k_2	k_3	k_4	k_2/k_1
0.7 (40°C)	Fig. 6	7.2×10^{-1}	1.6	7×10^{-2}	3×10^{-1}	2.2
	Fig. 7	9.0×10^{-1}	2.2	3×10^{-1}	8×10^{-2}	2.4
9.6 (40°C)	*a*)	5.6×10^{-1}	1.3	2×10^{-1}	2×10^{-2}	2.3
	b)	5.0×10^{-1}	1.1	9×10^{-2}	3×10^{-2}	2.2
6.9 (50°C)	*a*)	1.5×10^{-2}	0.025	8×10^{-4}	5×10^{-3}	1.7
	b)	2.6×10^{-2}	0.042	2×10^{-3}	8×10^{-3}	1.6

a) Semilogarithmic plots analogous to Fig. 6.
b) Semilogarithmic plots analogous to Fig. 7.

chin in a degradation path other than that shown in Chart 1. Further work is required to determine the actual reason for the difference.

Conclusion

It was found that the degradation of KB-53 in aqueous solution consisted of the following reactions, which were enormously dependent on pH. (1) Autoxidation: this easily occurred in a basic aqueous solution not flushed with nitrogen. (2) Dimerization: this easily occurred in a strongly acidic aqueous solution and obeyed apparent second-order kinetics. (3) Epimerization: this easily occurred as the pH increased, and the reverse reaction between KB-53 and epicatechin produced by epimerization took place especially in basic aqueous solution.

Further quantitative studies on the characteristics of these degradation reactions are in progress.

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