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Degradation of (+)-Cyanidanol-3 by Sodium Sulfite in Aqueous Solution[1)]

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The reaction of (+)-cyanidanol-3 with sodium sulfite in aqueous solution was investigated. (+)-Cyanidanol-3 was degraded by sodium sulfite in aqueous solution to afford a water-soluble degradation product, which was assumed to be (+)-cyanidanol-3 carrying the sodium sulfonate function in place of the aliphatic hydroxy group at the C-3 position. Further, it was found that the rate of degradation of (+)-cyanidanol-3 by sodium sulfite was approximately the same as that of epicatechin, that the rate was proportional to the concentration of sodium sulfite and to the sum of the concentrations of (+)-cyanidanol-3 and sodium sulfite, and that (+)-cyanidanol-3 or epicatechin was degraded more rapidly by sodium sulfite as the pH was increased. The activation energy of the degradation was calculated to be 28 kcal/mol at pH 8.0 from Arrhenius plots.

Keywords——(+)-cyanidanol-3; stability; degradation; kinetics; epicatechin; sodium sulfite; hepatoprotector

(+)-Cyanidanol-3 ("Cianidanol") is one of the flavans obtained from wood and leaves of *Uncaria gambir*, and recently its hepatoprotective activity has been reported by Hennings.[2)] In previous work,[3)] we studied the stability of Cianidanol in aqueous solution in order to develop liquid dosage forms, and found that severe autoxidation of Cianidanol occurred in basic solution not flushed with nitrogen. When this hepatoprotector is manufactured in liquid dosage forms, the addition of an antioxidant is therefore required for inhibition of the autoxidation of Cianidanol in solutions. Sodium bisulfite, sodium sulfite, ascorbic acid, thioglycerol and so on are commonly used as antioxidants[4)] for aqueous pharmaceutical liquids. Sodium bisulfite, one of these antioxidants, is well known to react reversibly or irreversibly with various functional groups in drug molecules such as aldehyde, ketone and alkene. The reactivity of sodium bisulfite with drugs has attracted attention since the inactivation of epinephrine by sodium bisulfite was reported by Higuchi *et al.*[5)] The reactivity of sodium bisulfite with dexamethasone-21-phosphate,[6)] morphine,[7)] fluorouracil[8)] and so on has been described. Sodium sulfite is also widely used as an antioxidant in solutions, even though its reactivity with drugs in aqueous systems has not been fully investigated. Rather little is known about the kinetics of degradation of drugs by sodium sulfite as well as bisulfite. In this report, we described the kinetics of degradation of Cianidanol by sodium sulfite, the chemical structure of the degradation product, the effect of pH on the degradation and the activation energy.

Experimental

Materials——Cianidanol was obtained from Zyma S. A. and recrystallized from water, then dried over phosphorus pentoxide. (±)-Epicatechin was obtained from Zyma S. A. as a standard sample and used as supplied. Other chemicals were of reagent grade quality. Deionized, then distilled water was used in all experiments.

Assay Methods——(a) The Stability of Cianidanol in Aqueous Solution containing Sodium Sulfite: Two hundred milligrams of Cianidanol was dissolved in 90 ml of phosphate buffer solution (0.03 M NaOH + 0.05 M KH_2PO_4 + 0.07 M NaCl) containing 2.0×10^{-3} M to 1.6×10^{-2} M sodium sulfite. Then the pH of the solution was adjusted to 6.9 with a small amount of 0.1 N hydrochloric acid and it was made up to 100 ml

with the phosphate buffer solution. Eleven milliliters of the solution was placed in a 10 ml glass ampule, sealed (the head space of the ampule was approximately 3 ml) and immersed in a controlled temperature water bath at 50±0.2°C. Ampules were withdrawn at appropriate intervals and cooled to room temperature. To 5 ml of the solution in the ampule was added 10 ml of ethyl acetate. The mixture was 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. Gas-liquid chromatography 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, 240 and 310°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 50 ml/min. Satisfactorily separate peaks of trimethylsilylated Cianidanol and epicatechin were detected as reported in the previous work.[3] Quantitation was based on peak height ratio comparison with linear standard plots.

(b) The Absorption Spectral Change of Cianidanol in Aqueous Solution on Autoxidation: As described in (a), 6.9×10^{-3} M Cianidanol solution containing sodium sulfite ranging from 2.1×10^{-3} to 3.2×10^{-2} M was heated at 50±0.2°C. The degraded solution in the ampule was diluted with phosphate buffer solution and the absorbance at 400 nm was measured. In addition, the absorption spectral change of the solution without sodium sulfite (control solution) was measured at 350—700 nm.

Kinetic Measurement of Degradation of Cianidanol by Sodium Sulfite——Equimolar amounts of Cianidanol and sodium sulfite (3×10^{-2} or 6×10^{-2} M) were dissolved in phosphate buffer solutions of pH 6.0, 6.9 and 8.0, $\mu=0.15$ (pH 6.0, 0.0057 M NaOH+0.05 M KH_2PO_4+0.094 M KCl; pH 6.9, 0.03 M NaOH+0.05 M KH_2PO_4+0.07 M NaCl; pH 8.0, 0.047 M NaOH+0.05 M KH_2PO_4+0.053 M KCl) and the pH values of the solutions were adjusted to 6.0, 6.9 and 8.0, respectively. Eleven milliliters of the solution was placed in a 10 ml glass ampule and sealed. The ampules were immersed in a controlled temperature water bath at 60±0.2°C. Ampules were withdrawn at appropriate intervals. The solution in the ampule was cooled to room temperature and diluted five times in the case of 3×10^{-2} M solution or ten times in the case of 6×10^{-2} M solution. Five milliliters of the diluted solution was treated as described in "Assay Methods" (a). In the case of the solution of pH 8.0, dilution was carried out with a mixture of the phosphate buffer solution and 0.1 N hydrochloric acid (10:1). With regard to the kinetics of the degradation of epicatechin by sodium sulfite, equimolar amounts of (±)-epicatechin and sodium sulfite (3×10^{-2} M) were dissolved in phosphate buffer solution of pH 8.0 and a kinetic run was carried out in a manner similar to that described for Cianidanol at pH 8.0.

Isolation of the Degradation Product——Cianidanol (7×10^{-2} M) and sodium sulfite (8×10^{-2} M) were heated in aqueous solution at 60°C and pH 6.9 for 48 h to isolate the degradation product generated by addition of sodium sulfite. The residue obtained from 150 ml of the degraded solution by evaporation was mixed with 250 ml of acetone–methanol mixture (1:4), and the whole was refluxed for 30 min and allowed to stand overnight at −10°C. After filtration, the mother liquor was concentrated, and the residue was dissolved in 100 ml of water and extracted with ethyl acetate to remove intact Cianidanol and epicatechin. Then the aqueous layer was concentrated and the residue was purified by column chromatography using cellulose powder (Whatman, CF-11) as a support and water as the eluent to afford the degradation product as a water-soluble pale yellow solid, yield 1.1 g, *Rf* 0.81, mp>350°C.

Acetylation of Isolated Degradation Product——Twenty-five milliliters of acetic anhydride, 0.2 ml of pyridine and 2.5 g of the isolated degradation product were mixed and heated at 60°C for 1 h to obtain a uniform solution. To this, 50 ml of water was added, and then the whole was stirred for a few min and concentrated. Ethyl acetate (300 ml) was added to 30 ml of the methanol solution of the residue, and the acetylated degradation product precipitated as a white solid, 2.6 g, mp 144—147°C (dec.).

Results and Discussion

Effect of Sodium Sulfite on the Autoxidation of Cianidanol

As shown in Fig. 1, the absorption of an aqueous solution of Cianidanol at around 400 nm increased as the solution produced a yellow to wine-red color after heating at 50°C and pH 6.9 (the absorption maximum of intact Cianidanol is at 280 nm). Sodium sulfite ranging from 2.1×10^{-3} to 3.2×10^{-2} M was added to Cianidanol solution and the absorbance at 400 nm was measured. As shown in Fig. 2, coloration of the solution and increase in absorbance at 400 nm were not observed when over 6.4×10^{-3} M sodium sulfite had been added. Consequently, it appeared that the autoxidation of Cianidanol in the ampule was sufficiently inhibited by adding 6.4×10^{-3} M sulfite.

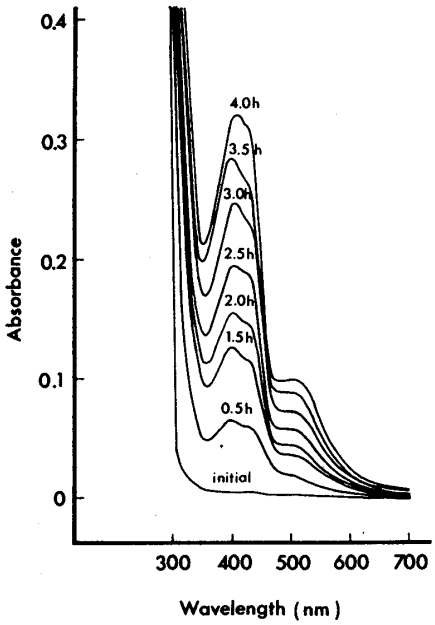


Fig. 1. Spectral Change of 6.9×10^{-3} M Cianidanol in Aqueous Solution as a Result of Autoxidaaion at 50°C and pH 6.9

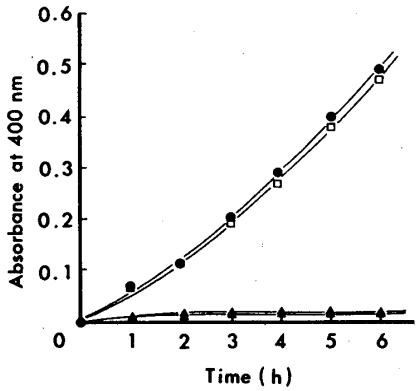


Fig. 2. Effect of Sodium Sulfite on the Autoxidation of 6.9×10^{-3} M Cianidanol in Aqueous Solution at 50°C and pH 6.9

—●— control, —□— Na_2SO_3 2.1×10^{-3} M, —△— Na_2SO_3 6.4×10^{-3} M, —△— Na_2SO_3 3.2×10^{-2} M.

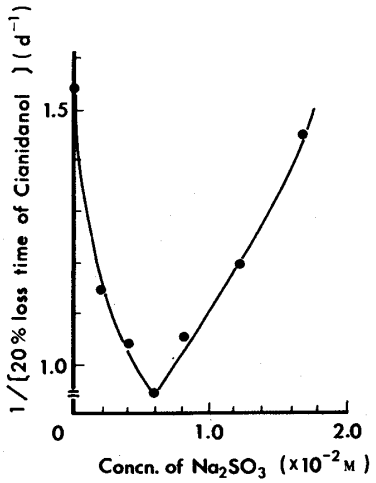


Fig. 3. Stability of 6.9×10^{-3} M Cianidanol in Aqueous Solution containing Sodium Sulfite at 50°C and pH 6.9

Stability of Cianidanol in Aqueous Solution containing Sodium Sulfite

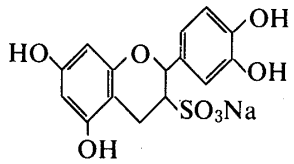
Aqueous solutions of Cianidanol (6.9×10^{-3} M) containing 2.0×10^{-3} to 1.6×10^{-2} M sodium sulfite were heated at 50°C and pH 6.9, and the change of remaining Cianidanol with time was measured according to "Assay Methods" (a). The 20% loss time of Cianidanol was measured graphically from the results and is plotted in Fig. 3 as its reciprocal versus concentration of sodium sulfite. As shown in Fig. 3, the stability of Cianidanol was improved by addition of sodium sulfite up to 6.0×10^{-3} M, while Cianidanol became more labile with increase in sodium sulfite concentration over 7.9×10^{-3} M. These results suggest that Cianidanol is degraded by sodium sulfite, since sodium sulfite was assumed to be an adequate antioxidant at a concentration as low as 6.4×10^{-3} M from the results shown in Fig. 2.

Identification of the Degradation Product generated by Addition of Sodium Sulfite

The degraded solution (initial concentrations of Cianidanol and sodium sulfite were 6.9×10^{-3} and 3.2×10^{-2} M, respectively) after being heated at 50°C and pH 6.9 for 24 h was subjected to thin-layer chromatography (TLC) on a cellulose plate (Cellulose F, Merck) using H_2O–dioxane–acetic acid (10:1:1) as the developing solvent. The products were detected by means of 10% Na_2CO_3 aq. and 0.5% Echtblausalz B (Merck) aq. spray. Two spots of degradation products were detected at *Rf* 0.50 and 0.81. The degradation product of *Rf* 0.50 was identical with epicatechin standard and seemed to be produced by epimerization of Cianidanol, as reported in the previous work.[3] The epimer produced by degradation of Cianidanol in aqueous solution was considered to be further degraded by sodium sulfite to give the degradation product of *Rf* 0.81. To confirm this, equimolar amounts of (±)-epicatechin and sodium sulfite (3×10^{-2} M) were heated in aqueous solution at pH 8.0 and 60°C for 8 h, and then the degraded solution was subjected to TLC. Two spots (*Rf* 0.64 and 0.81) other than the spot of epicatechin (*Rf* 0.50) were detected and their *Rf* values were identical with that of Cianidanol and the degradation product of Cianidanol produced by sodium sulfite, respectively.

Qualitative analysis of the isolated degradation product was carried out as follows. After carbonization of the degradation product, water was added to the residue, and then the whole

was stirred and filtered. An aqueous solution of barium chloride was added to the mother liquor to produce a white precipitate. This precipitate was insoluble in dilute nitric acid. In addition, the presence of sodium salt was revealed by the flame reaction. These results suggested that a sodium sulfonate function was present in the degradation product. Since the signal of the aliphatic hydroxy group of Cianidanol at C-3 was not detected in the nuclear magnetic resonance (NMR) spectrum of the degradation product, as shown in Table I, the chemical structure of the degradation product, was assumed to be Cianidanol containing the sodium sulfonate function in place of the aliphatic hydroxy group at the C-3 position. Next, the degradation product was acetylated as described in the experimental section. The NMR data for the acetylated degradation product are shown in Table I together with those for acetylated Cianidanol. Recently, the formation of (+)-epicatechin-2-sulfonate by the reaction of Cianidanol with sodium bisulfite in aqueous solution was reported by Courbat *et al.*[9] However, the chemical structure deduced from the NMR spectrum of the degradation product in our study was not identical with (+)-epicatechin-2-sulfonate, even though the reaction conditions were very similar to those described in the patent. That is to say, the signal of the hydroxy proton at the C-3 position of Cianidanol, which would disappear on addition of D_2O, was not detected in the NMR spectrum of the degradation product, as shown in Table I. Further, the signal of the acetyl methyl protons of acetylated Cianidanol at the C-3 position, which would be detected if the aliphatic hydroxy group remained unchanged in the degradation product, was not detected in the NMR spectrum of the acetylated degradation product. Consequently, the chemical structure of the degradation product of Cianidanol by sodium sulfite was assumed to be as follows:



Further investigations relating to the stereochemistry of the degradation product are in progress.

TABLE I. NMR Spectral Data for Cianidanol, Degradation Product, Acetylated Cianidanol and Acetylated Degradation Product (δ Values, ppm)[a]

	4-CH_2	3-CH	2-CH	3-OH	6-Arom.	8-Arom.	2',5',6'-Arom.	Phenolic OH
Cianidanol (in DMSO-d_6)	2.24—2.80 (2H, m)	3.56—4.00 (1H, m)	4.44 (1H,d, J=8)	4.72—5.00[b] (1H, m)	5.64 (1H,d, J=4)	5.84 (1H,d, J=4)	6.40—6.80 (3H, m)	8.40—9.40[b] (4H,br s)
Degradation product (in DMSO-d_6)	2.05—2.75 (2H, m)	4.04—4.26 (1H, m)	3.44 (1H,d, J=8)	n.d.[c]	5.72 (2H, s)		6.32—7.08 (3H, m)	8.20—9.00 (4H,br s)

	3-Acetyl CH_3	5,7,3',4'-Acetyl-CH_3	4-CH_2	2-CH	3-CH	6-Arom.	8-Arom.	2',5',6-Arom.
Acetylated cianidanol (in $CDCl_3$)	1.98 (3H, s)	2.24 (12H, s)	2.52—3.08 (2H, m)	5.13 (1H,d, J=6)	5.18—5.40 (1H, m)	6.58 (1H,d, J=2)	6.65 (1H,d, J=2)	7.04—7.26 (3H, m)
Acetylated degradation product (in $CDCl_3$)	n.d.[c]	2.00—2.64[d] (14H, m)		3.90 (1H,d, J=6)	4.00—4.40 (1H, m)	6.82 (2H, s)		7.10—7.50 (3H, m)

a) Measured at 100 MHz with TMS as an internal standard, J values are expressed in Hz.
b) Not detected after addition of D_2O.
c) Not detected.
d) Signals overlapped.

Effect of pH on the Degradation of Cianidanol by Sodium Sulfite

To investigate the effect of pH on the degradation of Cianidanol by sodium sulfite, equimolar amounts of Cianidanol and sodium sulfite (6×10^{-2}M) were dissolved in buffer solu-

tions of pH 6—8, and the solutions were flushed with nitrogen and heated at 60°C. As shown in Fig. 4, only epimerization of Cianidanol to epicatechin occurred, and the sum of the concentrations of Cianidanol and epicatechin did not vary in the control solutions at pH 6.0, 6.9 and 8.0 (only the result obtained at pH 6.0 is shown in Fig. 4). On the other hand, the sum of the concentrations of Cianidanol and epicatechin decreased increasingly rapidly with increase in pH value in the case of the solution containing sodium sulfite.

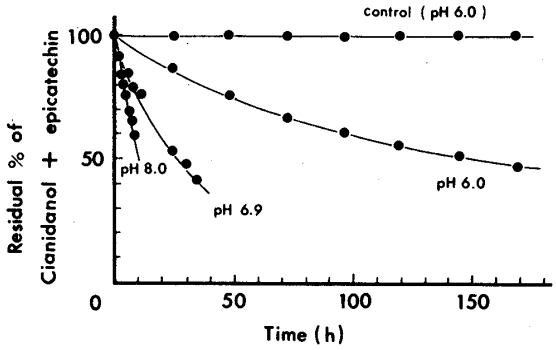


Fig. 4. Effect of pH on the Degradation of Cianidanol in Aqueous Solution containing Sodium Sulfite at 60°C

Initial conc. of Cianidanol, 0.060M; initial conc. of Na_2SO_3, 0.060M; control, without Na_2SO_3 at pH 6.0.

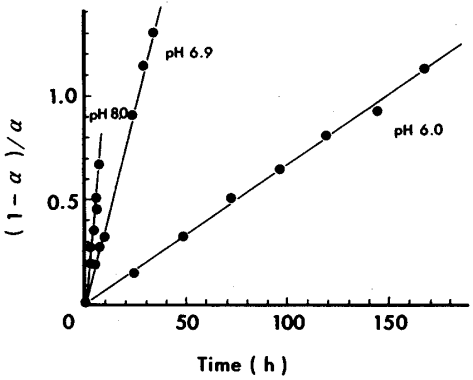


Fig. 5. Second-order Plots of the Degradation Reaction of Cianidanol with Sodium Sulfite

Kinetic Studies

Since epicatechin was degraded by sodium sulfite in the same manner as Cianidanol, as mentioned above, if the rate constant of the degradation of epicatechin by sodium sulfite (k) is the same as that of Cianidanol in the following scheme,

$$\text{degradation product} \xleftarrow[Na_2SO_3]{k} \text{Cianidanol} \rightleftarrows \text{epicatechin} \xrightarrow[Na_2SO_3]{k} \text{degradation product}$$

the rate of degradation of Cianidanol or epicatechin can be expressed by equation (1).[10]

$$\frac{-d[\text{Cianidanol} + \text{epicatechin}]}{dt} = k[\text{Cianidanol} + \text{epicatechin}][Na_2SO_3] \quad (1)$$

where [Cianidanol+epicatechin] is the sum of the molar concentrations of Cianidanol and epicatechin at time t, $[Na_2SO_3]$ is the molar concentration of sodium sulfite at time t, and k is the rate constant of the degradation of epicatechin or Cianidanol by sodium sulfite.

When the initial concentration of [Cianidanol+epicatechin], that is [Cianidanol+epicatechin]$_0$, is equal to the initial concentration of sodium sulfite, the rate constant (k) is given by equation (2),

TABLE II. Rate Constants of 2nd-order Reaction at 60℃

Initial conc. (M)		pH	k
Cianidanol	Na_2SO_3		($M^{-1}h^{-1}$)
0.031	0.031	6.0	0.096
0.064	0.064	6.0	0.10
0.030	0.030	6.9	0.57
0.063	0.063	6.9	0.61
0.065	0.065	8.0	1.4
Epicatechin			
0.031	0.031	8.0	1.3

$$(1-\alpha)/\alpha=[\text{Cianidanol + epicatechin}]_0 kt \quad (2)$$

where $\alpha=$[Cianidanol+epicatechin]/[Cianidanol+epicatechin]$_0$. As shown in Fig. 5, plots of $(1-\alpha)/\alpha$ against time at each pH gave a straight line on the basis of the data shown in Fig. 4. From the slopes, the rate constant at each pH was calculated and the results are shown in Table II. As shown in Table II, the rate constants derived from the solutions in which the initial concentrations of Cianidanol or sodium sulfite were 0.03 M and 0.06 M were nearly equal at pH 6.0 or 6.9. Moreover, the rate constant derived from the solution in which epicatechin and sodium sulfite had been dissolved at pH 8.0 was very close to that derived from the solution containing Cianidanol and sodium sulfite. It is suggested from these results that the reactivity of epicatechin with sodium sulfite is the same as that of Cianidanol and that the kinetics of the degradation are well expressed by equation (1).

Next, equimolar amounts of Cianidanol and sodium sulfite (6.5×10^{-2} M were heated at 40, 50 and 60°C in the buffer solution of pH 8.0. Plots of $(1-\alpha)/\alpha$ against time at each temperature gave a straight line as shown in Fig. 6. Arrhenius plots of the rate constants obtained from these slopes gave a straight line, and the apparent activation energy of the degradation reaction at pH 8.0 was calculated to be 28 kcal/mol (Fig. 7). Both the reaction[11] between epinephrine and bisulfite or bisulfite ion and the reaction[12] between salicyl alcohol and bisulfite ion are known to exhibit a somewhat smaller activation energy (24 kcal/mol). This difference seems to suggest that the degradation of Cianidanol by sodium sulfite involves direct attack of sulfite ion not on Cianidanol but on an intermediate such as a dissociated form of Cianidanol. However, further work is required to confirm this.

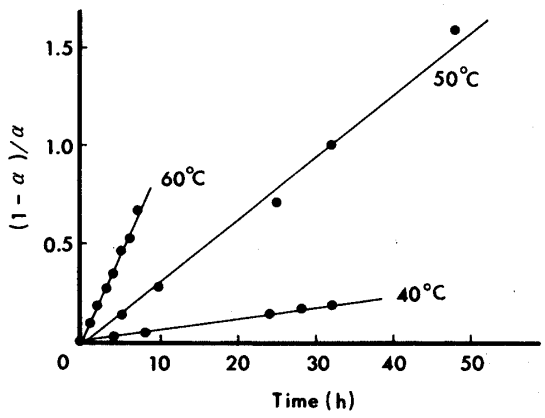


Fig. 6. Second-order Plots of the Degradation Reaction of Cianidanol with Sodium Sulfite at pH 8.0

Initial conc. of Cianidanol, 0.065M; initial conc. of Na_2SO_3, 0.065M.

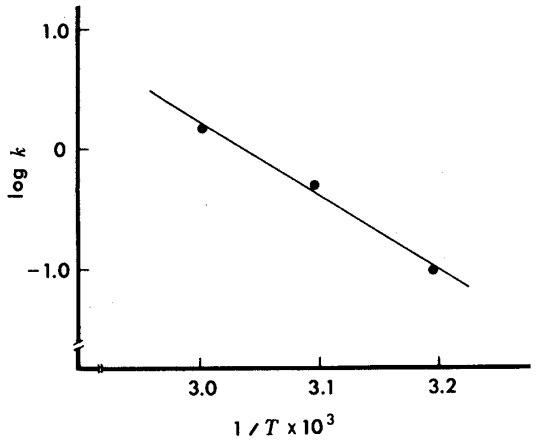


Fig. 7. Arrhenius Plots based on the Rate Constants measured at pH 8.0

Conclusion

Cianidanol is degraded by sodium sulfite in aqueous solution to afford a water-soluble degradation product, which was concluded to be Cianidanol in which a sodium sulfonate moiety replaces the aliphatic hydroxy group at the C-3 position. It was proved that the rate of degradation of Cianidanol by sodium sulfite is approximately the same as that of epicatechin, that the rate is proportional to the concentration of sodium sulfite and the sum of the concentrations of Cianidanol and epicatechin, and that Cianidanol or epicatechin is degraded increasingly rapidly by sodium sulfite with increase in pH.

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References and Note

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