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Kinetic Studies on Decomposition of Glutathione. II.[1] Anaerobic Decomposition in Aqueous Solution

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Kinetics studies were performed on the non-enzymatic decomposition of glutathione (GSH) in anaerobic aqueous solution. The apparent first-order rate constants of decomposition were determined in the pH range of 1—12, ionic strength 0.5, at 60° and the pH profile was constructed from the buffer-free rate constants and pH values. The decomposition of GSH took place rapidly in acidic solution, but the rate constant showed a minimum in the pH range of 5—6, a maximum at pH 8.5 and a plateau in the pH range of 10—12. The effects of ionic strength and dielectric constant were almost negligible. The apparent activation energies of decomposition of GSH were all in the range of 19—21 kcal/mol. The rate constants of decomposition were related to the mole fractions of ionic species of GSH, and could be expressed as a function of hydrogen ion activity at arbitrary pH.

Keywords——glutathione; kinetics; decomposition; dissociation constant; activation energy; rate constant

Many kinetic studies on the enzymatic decomposition of glutathione (GSH) have been reported, but few deal with its non-enzymatic decomposition in anaerobic aqueous solution. Matsuki *et al.*[3] reported that GSH was most stable in anaerobic solution at pH 4.5—7 and that its decomposition was accelerated at pH 4 and pH 9, so that acid-base catalysis was presumably involved. The kinetic features of the decomposition of GSH in solution nevertheless remained obscure, and may be complicated because of the presence of four dissociating groups (two carboxyl groups, one amino group and a sulfhydryl group) in GSH.

We report here a detailed advanced kinetic study on the non-enzymatic decomposition of GSH in anaerobic aqueous solution.

Experimental

Materials——Crystalline GSH (Yamanouchi Pharmaceutical Co.) was recrystallized from 50% ethanol.[4] All other chemicals used were of the highest grade commercially available.

Kinetic Procedures——An accurately weighed quantity of GSH was dissolved in an oxygen-free acid, alkali or appropriate buffer solution bubbled through with N_2 gas. The initial concentration of GSH in the reaction solutions was 3×10^{-3} M or 10^{-2} M. The solutions were adjusted to an ionic strength of 0.5 with potassium chloride. Each buffer solution was made up to 0.05, 0.10 and 0.15 M (total buffer concentration). These buffer solutions were citrate buffer (pH 2.4—2.8), acetate buffer (pH 3.2—5.0), phosphate buffer (pH 5.4—7.8) and borate buffer (pH 8.4—11.0). At pH 1—2, the pH of reaction solutions was adjusted with HCl and at pH 11—12, with NaOH. The pH of the reaction solution was determined at the experimental temperature with N_2 gas bubbling through the solution. The reaction solution was poured into ampoules, which were sealed under nitrogen. The ampoules containing the reaction solution were allowed to stand in a constant temperature bath that was regulated to ±0.1°. Ampoules were removed at appropriate intervals and the contents analyzed.

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1) Part I: M. Aruga, S. Awazu, and M. Hanano, *Chem. Pharm. Bull.*, **26**, 2081 (1978).

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3) T. Matsuki and Y. Sumi, *Nippon Nogeikagaku Kaishi*, **47**, 185 (1973).

4) V. du Vigneaud and G.L. Miller, *Biochem. Preps.*, **2**, 91 (1952).

Determination of GSH[5]——The GSH-*o*-phthalaldehyde method was applied to determine GSH specifically.

Determination of pK_a——The apparent pK_a values of GSH were determined by potentiometric titration at an ionic strength of 0.5 at 60°. pK_1 and pK_2 were estimated by the method of Pirie *et al.*,[6] and pK_3 and pK_4 by the method of Li *et al.*[7]

Results and Discussion

Decomposition Rate Constants

Figures 1, 2, and 3 illustrate the time courses of decomposition in 0.05 M buffer, ionic strength 0.5, at 60°. In these cases, the initial concentration of GSH in the solution was 3×10^{-3} M. In the range of pH 1—10.5, the semi-logarithmic plots of the ratio of remaining GSH *vs.* reaction time at various pH values are almost linear, as shown in Fig. 1, 2 and 3. This indicates that the decomposition of GSH is an apparent first-order reaction in this range of pH. The pseudo-first order rate constants, k_{obs}, were calculated by the least-squares method from these data. In the range of pH 11—12 at the early stage of decomposition of GSH, the semi-logarithmic plots are almost linear (Fig. 3), while after the early stage the decomposition rate constant apparently decreases as the decomposition proceeds. In these cases, the pseudo-first order rate constants were calculated at the early stage where the plots are linear. The time courses of decomposition in the cases of 0.10 and 0.15 M buffer solution showed similar linearity. Based on these experimental results we concluded that the decomposition of GSH could be analyzed as a first-order reaction.

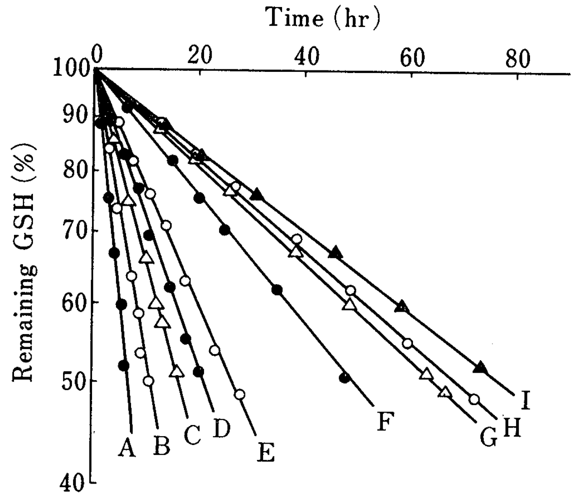

Fig. 1. Time Courses of Anaerobic Decomposition of Glutathione in Aqueous Solution (60°, $\mu=0.5$, buffer concentration; 0.05 M)

—●—A; pH 1.00, —○—B; pH 1.50, —△—C; pH 1.96, —●—D; pH 2.43, —○—E; pH 2.84; —●—F; pH 3.22, —△—G; pH 3.71, —○—H; pH 3.99, —▲—I; pH 4.43.

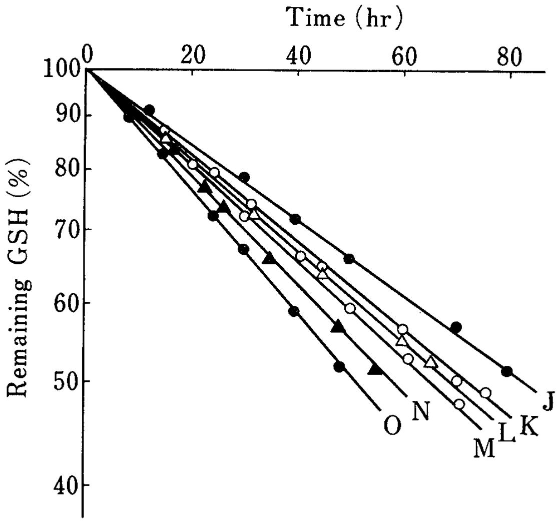

Fig. 2. Time Courses of Anaerobic Decomposition of Glutathione in Aqueous Solution (60°, $\mu=0.5$, buffer concentration; 0.05 M)

—●—J; pH 5.02, —○—K, pH 5.36, —△—L; pH 5.61, —○—M; pH 6.09, —▲—N; pH 6.50, —●—O; pH 7.01.

Effect of Buffer Concentration

The plots of rate constants *vs.* buffer concentrations at constant ionic strength were linear at each pH, as shown in Fig. 4. The effect was marked in phosphate buffer but only slight in borate buffer. The buffer-free rate constants, k_{tot}, at various pH values were

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[5] V.H. Cohn and J. Lyle, *Anal. Biochem.*, **14**, 434 (1966).

[6] N.W. Pirie and K.G. Pinkey, *J. Biol. Chem.*, **84**, 321 (1929).

[7] N.C. Li, O. Gawron, and G. Bascaus, *J. Am. Chem. Soc.*, **76**, 225 (1954).

calculated by extrapolation of the data shown in Fig. 4. These buffer effects indicate that GSH decomposition involves general acid–base catalysis.

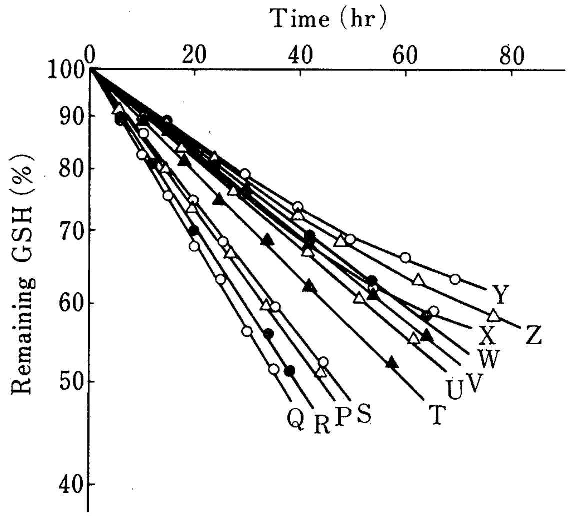

Fig. 3. Time Courses of Anaerobic Decomposition of Glutathione in Aqueous Solution (60°, μ=0.5, buffer concentration; 0.05 M)

—△—P; pH 7.40, —○—Q; pH 7.81, —●—R; pH 8.43, —○—S; pH 8.91, —▲—T; pH 9.37, —△—U; pH 9.69, —▲—V; pH 10.00, —●—W; pH 10.48, —○—X; pH 10.99, —○—Y; pH 11.50, —△—Z; pH 12.00.

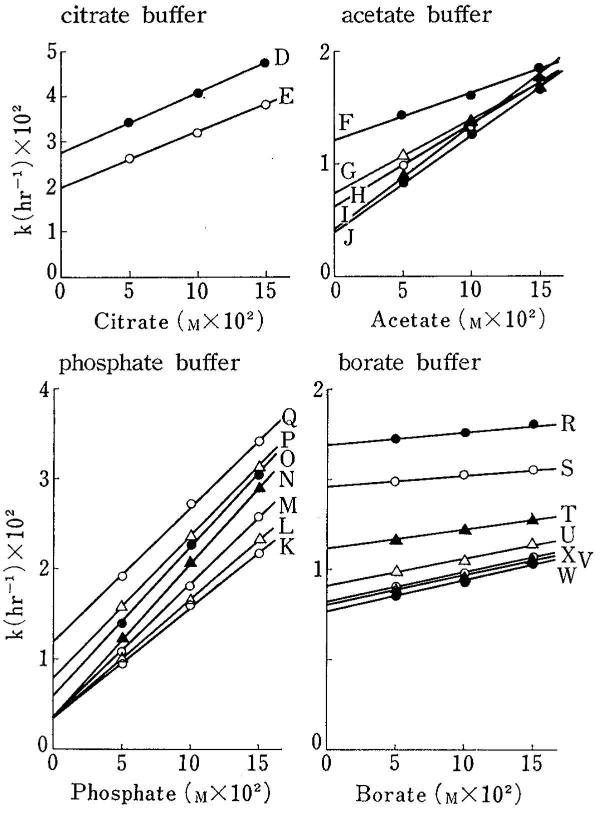

Fig. 4. Effect of Buffer Concentration on the Decomposition of Glutathione

Symbols; see Fig. 1, 2, and 3.

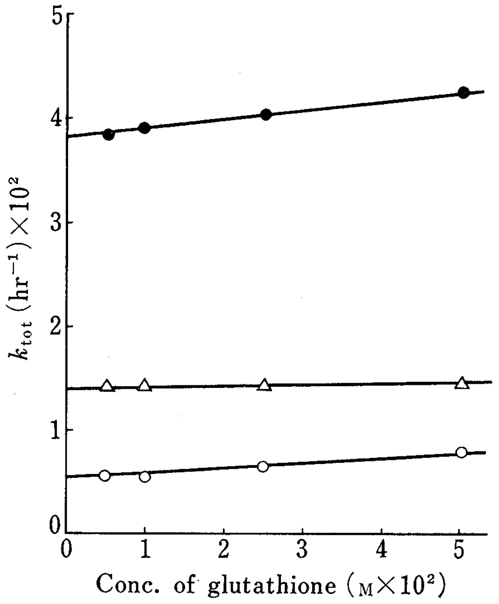

Fig. 5. Effect of Initial Concentration on the Decomposition Rate Constants of Glutathione

—●—; pH 2.00, —△—; pH 9.00, —○—; pH 4.00.

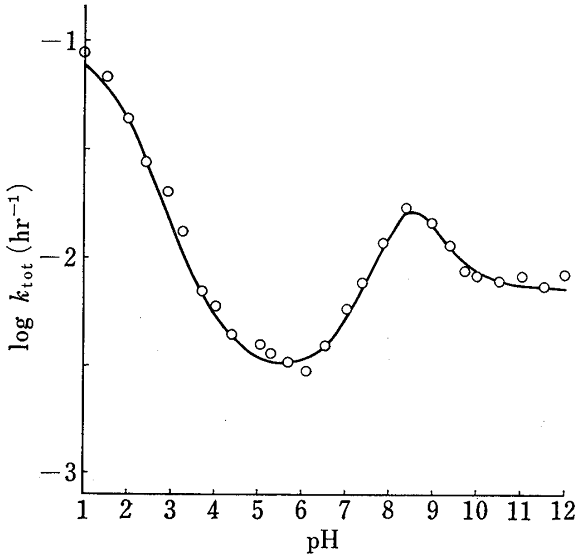

Fig. 6. log (Decomposition Rate Constant)–pH Profile (60°, μ=0.5)

——; Calculated from equation (7),
○; Experimental data.

Effect of GSH Concentration

When the concentration of GSH in solution was comparatively high, its effect on the decomposition rate was reported to be great.3) In the cases of low concentrations, of the order of 10^{-2} M, the effect of the concentration is slight and linear, as shown in Fig. 5. The rate constant at the initial concentration of 3×10^{-3} M is approximately equal (within experimental error) to the rate constant at extremely low concentrations. k_{tot} in this study, therefore, may be independent of the initial concentration.

pH Profile of the Rate Constants

The log k_{tot}–pH profile (Fig. 6) was constructed from the pseudo-first orden rate constants and pH at ionic strength 0.5 at 60°. The decomposition of GSH is rapid in acidic solution, and the rate decreases as the pH rises, reaching a minimum in the pH 5 to 6 region. Above pH 6, the rate increases as the pH rises, and reaches a maximum at pH 8.5, Above pH 8.5, the rate decreases, and the rate constant reaches a plateau in the pH 10 to 12 region.

Effect of Temperature

The temperature dependence of the decomposition of GSH was estimated by determining the pseudo-first order rate constants at various temperatures at a constant ionic strenth of 0.5. As shown in Fig. 7, Arrhenius plots of log k_{tot} *vs* $1/T$ at each pH were linear. The apparent activation energies calculated from the slopes of the linear plots are shown in Table I. Although the decomposition reaction of GSH may involve different reaction species and catalysts in each pH region, the activation energy was about 20 kcal/mol under all conditions.

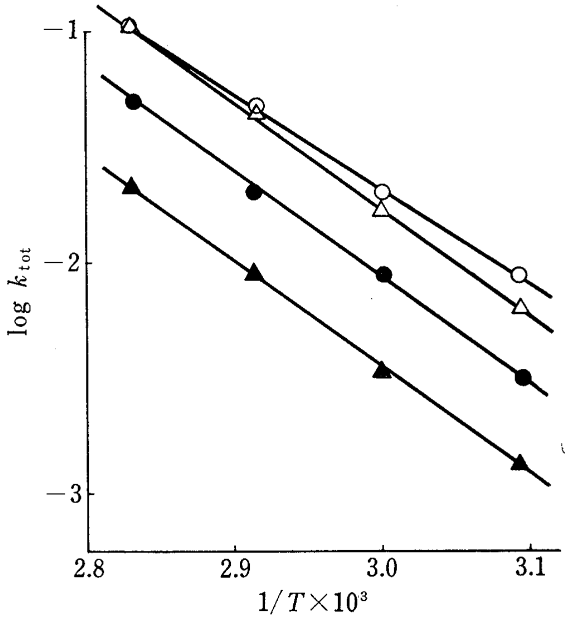

Fig. 7. Temperature Dependence of Glutathione Decomposition in Aqueous Solution

—○—; pH 2.83, —▲—; pH 5.56, —△—; pH 8.43, —●—; pH 10.00.

Primary Salt Effect

Primary salt effect on the decomposition of GSH was investigated at 60° with unbuffered reaction solutions in which the initial concentration of GSH was 10^{-2} M. The ionic strength of the solutions ranged from 0.02 to 0.30 (adjusted by the addition of potassium chloride at pH 3.00, 5.55, 8.50 and 10.50). At each pH, the pH shifted slightly as decomposition of GSH proceeded; in the ionic strength range of 0.02—0.30, the pH shift in the reaction solution could be taken as the same, regardless of ionic strength. It was, therefore, possible to investigate the primary salt effect on the decomposition of GSH

Table I. Apparent Activation Energies of Glutathione Decomposition in Anaerobic Aqueous Solutions

	Activation energy (kcal/mol)
pH 2.84	18.9
pH 5.56	20.9
pH 8.43	21.2
pH 10.00	20.8

by comparison of the decomposition ratios at the same time in unbuffered reaction solutions. Fig. 8 shows the time courses of GSH decomposition at ionic strength 0.02 and 0.30 at each pH. Those at ionic strengths of 0.04, 0.07 and 0.10 are almost the same as those at 0.02 or 0.30. Fig. 9 shows the average relative ratio of decomposed GSH at each time of determination as the function of ionic strength. It is clear that there is no change at each pH. Over the range of pH 3—10.5, therefore, it may be concluded that ionic strength only slightly effects the decomposition rate of GSH.

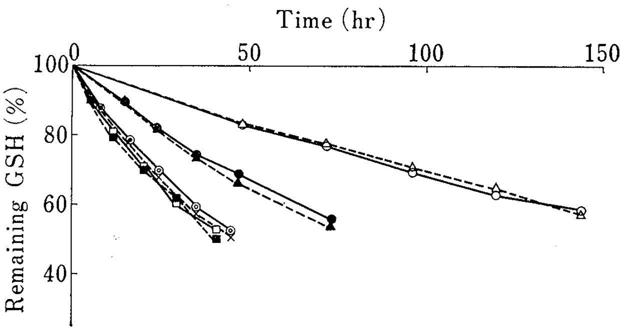

Fig. 8. Time Courses of Glutathione Decomposition in Unbuffered Solution at 60°, μ= 0.02 and 0.30

—○—; pH 3.00, μ=0.02, ---△---; pH 3.00, μ=0.30,
—◎—; pH 5.55, μ=0.02, ---×---; pH 5.55, μ=0.30,
—□—; pH 8.50, μ=0.02, ---■---; pH 8.50, μ=0.30,
—●—; pH 10.50, μ=0.02, ---▲---; pH 10.00, μ=0.30.

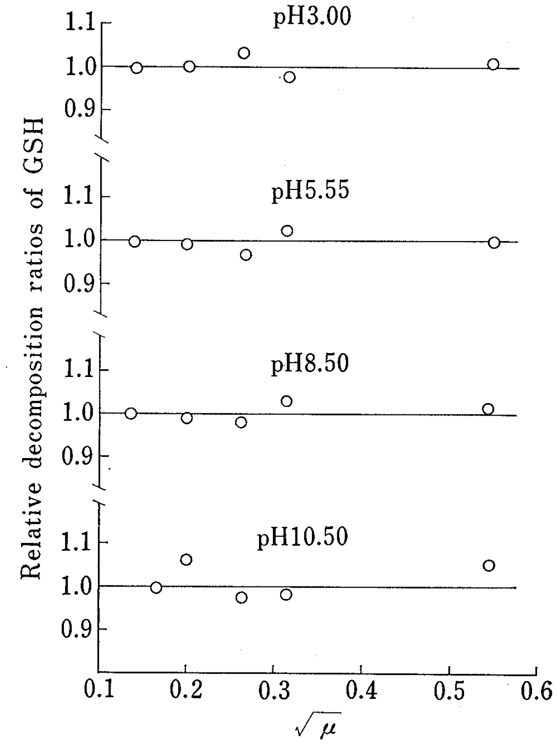

Fig. 9. Effect of Ionic Strength (μ) on the Rate of Glutathione Decomposition

Effect of Ethanol

The effect of ethanol on the decomposition of GSH was investigated at 60° with an unbuffered reaction solution containing ethanol. The initial concentration of GSH in solutions was 10^{-2} M. Ethanol was purified as described by Åkeröf.[8] The concentrations of ethanol were 0, 10, 30 and 45 w/w%, ionic strength 0.5, at pH 3.03, 5.51 and 8.55. At each pH, the pH shifted slightly as the decomposition of GSH proceeded. However, in the ethanol concentration range of 0—45%, the pH shifts could be taken as the same regardless of the ethanol concentration, so that it was possible to investigate the effect of ethanol on the decomposition of GSH by comparison of decomposition ratios at the same reaction time in unbuffered reaction solutions. Fig. 10 shows the time courses of GSH decomposition in the cases of 0 and 45% ethanol at each pH. Those in 10 and 30% ethanol were almost the same as that in the absence of ethanol. Fig. 11 shows the average relative decomposition ratios of GSH at each time of determination as a function of ethanol concentration in the reaction solution. As shown in Fig. 11, at pH 3.03 and 5.51 the relative decomposition ratio of GSH does not vary as the ethanol concentration increases. At pH 8.5, however, the ratio decreases slightly as the ethanol concentration increases. The dielectric constants of the reaction solutions containing 0, 10, 30 and 45% ethanol are 66.6, 61.5, 51.0 and 43.2

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8) G. Åkeröf, *J. Am. Chem. Soc.*, **54**, 4125 (1932).

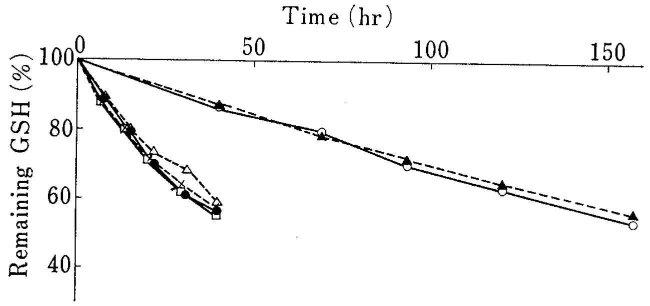


Fig. 10. Time Courses of Glutathione Decomposition in 0 and 45% Ethanol Solution at 60°

—●—; pH 3.03, 0% ethanol, ---△---; pH 3.03, 45% ethanol,
—○—; pH 5.51, 0% ethanol, ---▲---; pH 5.51, 45% ethanol,
—□—; pH 8.55 0% ethanol. ---×---; pH 8.55 45% ethanol.

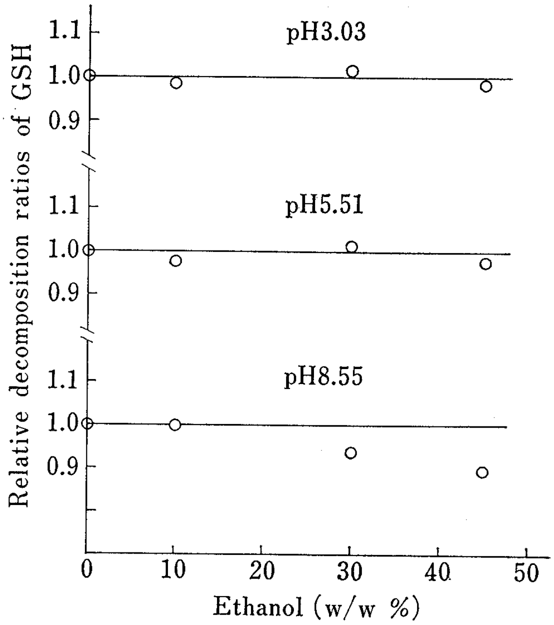
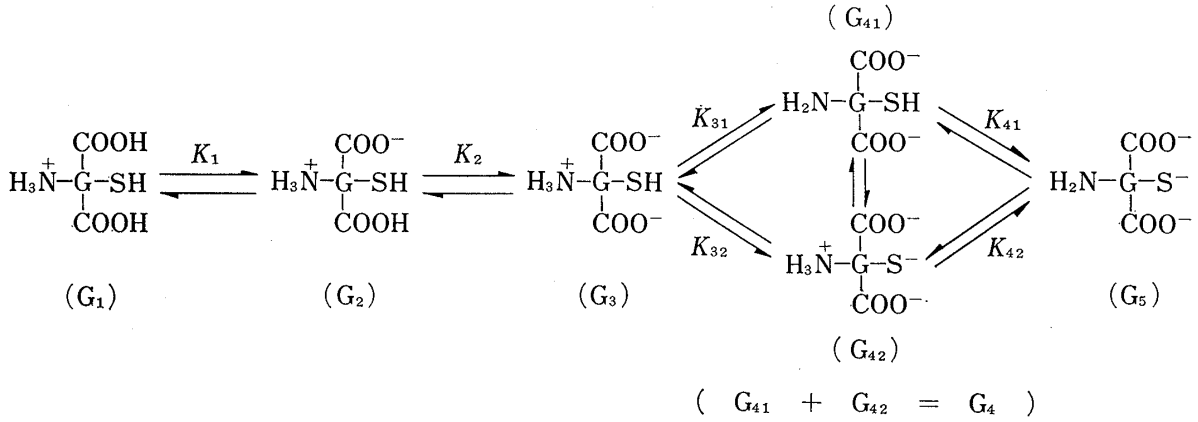


Fig. 11. Effect of Ethanol Concentration on the Decomposition of Glutathione



Scheme 1

at 60°, respectively, as calculated from the data reported by Akerlof.8) The effect of dielectric constant on the decomposition of GSH is considered to be slight.

Relationship between the Dissociation of GSH and Decomposition Rate Constants

GSH dissociates into ions in aqueous solution as shown in Scheme 1, where G_2 includes both G_{21} (HOOC-glu-cys-gly-COO$^-$) and G_{22} ($^-$OOC-glu-cys-gly-COOH), but the mole fraction of G_{22} is much larger than that of G_{21}. It was impossible to determine K_{31}, K_{32}, K_{41} and K_{42} by the usual potentiometric titration method. To the apparent dissociation constant K_3 between G_3 and G_4 (the sum of G_{41} and G_{42}) was determined. The apparent dissociation constant K_4 between G_4 and G_5 was also determined. The apparent dissociation constants of GSH at an ionic strength of 0.5 at 60° are shown in Table II.

The mole fractions of G_1, G_2, G_3, G_4, and G_5 at arbitrary pH are expressed approximately by equations (1)—(5), respectively, where FG_1, FG_2, FG_3, FG_4 and FG_5 represent the mole fractions of G_1—G_5, and a_H represents hydrogen ion activity.

TABLE II. Dissociation Constants of Glutathione

Dissociation constants (60°, ionic strength $\mu=0.50$)			
pK_1	pK_2	pK_3	pK_4
2.00	3.51	8.14	8.85

TABLE III. Obtained Parameters of Equation (6)

X_1	X_2	X_3	X_4	X_5
8.18×10^{-2}	1.23×10^{-2}	3.28×10^{-3}	2.63×10^{-2}	7.39×10^{-3}

(unit; hr^{-1})

$$\mathrm{FG}_1 = a_{\mathrm{H}}^4/(a_{\mathrm{H}}^2+K_1a_{\mathrm{H}}+K_1K_2)(a_{\mathrm{H}}^2+K_3a_{\mathrm{H}}+K_3K_4) \quad (1)$$

$$\mathrm{FG}_2 = K_1a_{\mathrm{H}}^3/(a_{\mathrm{H}}^2+K_1a_{\mathrm{H}}+K_1K_2)(a_{\mathrm{H}}^2+K_3a_{\mathrm{H}}+K_3K_4) \quad (2)$$

$$\mathrm{FG}_3 = K_1K_2a_{\mathrm{H}}^2/(a_{\mathrm{H}}^2+K_1a_{\mathrm{H}}+K_1K_2)(a_{\mathrm{H}}^2+K_3a_{\mathrm{H}}+K_3K_4) \quad (3)$$

$$\mathrm{FG}_4 = K_1K_2K_3a_{\mathrm{H}}/(a_{\mathrm{H}}^2+K_1a_{\mathrm{H}}+K_1K_2)(a_{\mathrm{H}}^2+K_3a_{\mathrm{H}}+K_3K_4) \quad (4)$$

$$\mathrm{FG}_5 = K_1K_2K_3K_4/(a_{\mathrm{H}}^2+K_1a_{\mathrm{H}}+K_1K_2)(a_{\mathrm{H2}}+K_3a_{\mathrm{H}}+K_3K_4) \quad (5)$$

The following relationship was assumed,

$$k_{\mathrm{tot}} = X_1\cdot\mathrm{FG}_1 + X_2\cdot\mathrm{FG}_2 + X_3\cdot\mathrm{FG}_3 + X_4\cdot\mathrm{FG}_4 + X_5\cdot\mathrm{FG}_5 \quad (6)$$

and the parameters, X_1, X_2, X_3, X_4 and X_5 were calculated by the least-squares method, by fitting equation (6) to the experimental rate constants at various pHs. The calculated values are shown in Table III. Substituting equations (1)—(5) into equation (6) with the calculated values of the parameters, X_1, X_2, X_3, X_4 and X_5, equation (7) is obtained for the rate constant as a function of hydrogen ion activity (a_{H}) at arbitrary pH.

$$k_{\mathrm{tot}} = \frac{8.18\times10^{-2}a_{\mathrm{H}}^4+1.23\times10^{-4}a_{\mathrm{H}}^3+1.01\times10^{-8}a_{\mathrm{H}}^2+5.57\times10^{-16}a_{\mathrm{H}}+2.34\times10^{-25}}{(a_{\mathrm{H}}^2+10^{-2}a_{\mathrm{H}}+3.09\times10^{-6})(a_{\mathrm{H}}^2+7.24\times10^{-9}a_{\mathrm{H}}+1.02\times10^{-17})} \quad (\mathrm{hr}^{-1}) \quad (7)$$

The solid line in Fig. 6 shows the values calculated from equation (7). The calculated values agree well with the experimental values, as shown in Fig. 6. It is thought that the decomposition rate constant of GSH in aqueous solution shows a minimum in the pH 5 to 6 region and a maximum at pH 8.5 because X_3, the parameter corresponding to the ionic species of G_3, is relatively small and X_4, the parameter corresponding to the ionic species of G_4, is relatively large.

Thus, the decomposition of GSH in aqueous solution over a wide range of pH can be understood in terms of the elementary reactions of ionic species of GSH in solution.