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Kinetic Studies on Decomposition of Glutathione. III.[1] Peptide Bond Cleavage and Desulfurization in Aqueous Solution

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Kinetic studies on the peptide bond (γ-glutamyl bond) cleavage and desulfurization of glutathione (GSH) in anaerobic aqueous solution were performed in the range of pH 1—12 at 60°. The desulfurization of GSH in neutral solution was very slow, but the rate increased markedly with increase of pH. In the range of pH 10—12, the pH of the reaction solution had little effect on the desulfurization rate. The cleavage rate of γ-glutamyl bond showed a minimum in the range of pH 5—6, a maximum at pH 8.5 and a plateau in the range of pH 10—12. The effects of ionic strength and dielectric constant on both γ-glutamyl bond cleavage and desulfurization were almost negligible. The apparent activation energy for cleavage of the γ-glutamyl bond was 19—21 kcal/mol and that for desulfurization was about 21 kcal/mol. The apparent first-order rate constants of both reactions 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. In the range of pH 6.5—10, the pH profile of the logarithmic cleavage rate constant of the γ-glutamyl bond was bell-shaped; it is likely that cleavage of the γ-glutamyl bond is accelerated, at least in the range of pH 7.5—10, by intramolecular catalysis involving the NH_2 and SH groups of GSH.

Keywords——glutathione; kinetics; decomposition; desulfurization; γ-glutamyl bond; dissociation constant; activation energy; rate constant

In the preceding paper,[1] kinetic studies on the non-enzymatic decomposition of glutathione (GSH) in anaerobic aqueous solution were reported. The decomposition of GSH is not a simple reaction, and under certain conditions peptide bond cleavage and desulfurization take place simultaneously.[3] The overall decomposition consists principally of these two reactions, so we sought to investigate the kinetics of these two decompositions. The peptide bond cleavage of GSH is particularly interesting in connection with the enzymatic decomposition of GSH.

This paper presents the results of our kinetic studies on the peptide bond cleavage reaction and the desulfurization reaction of GSH in anaerobic aqueous solution.

Experimental

Materials and Kinetic Procedures——The materials and kinetic procedures were the same as reported in the preceding paper.[1]

Assay of GSH——The GSH-*o*-phthalaldehyde method was used to determine GSH specifically.[4]

Estimation of Reducing Power——A sample of GSH solution was withdrawn and 0.1 N HCl was added to make the concentration of GSH 3×10^{-4}—4×10^{-4} M. N_2 gas was bubbled through the diluted solution for 10—15 minutes to exclude from the solution hydrogen sulfide produced by decomposition. Next, 2.5 ml of the solution was pipetted into a beaker and 5 ml of 0.1 N HCl was added. It was cooled to 0—7° in an ice-bath, then 0.5 ml of 5% KI solution and starch reagent were added, and the solution was titrated with 0.01 N KIO_3 in an ice-bath.[5]

1) Part II: M. Aruga, S. Awazu, and M. Hanano, *Chem. Pharm. Bull.*, **28**, 514 (1980).
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3) T. Matsuki and Y. Sumi, *Nippon Nogeikagaku Kaishi*, **47**, 185 (1973).
4) V.H. Cohn and J. Lyle, *Anal. Biochem.*, **14**, 434 (1963).
5) Y. Kuroiwa, *Nippon Nogeikagaku Kaishi*, **25**, 93 (1951).

Results and Discussion

Time Course of Decrease in Reducing Power (Desulfurization)

The time courses of decrease in reducing power for GSH anaerobic aqueous solution (0.05 M buffer) in the range of pH 7—12 at ionic strength 0.5 are shown in Fig. 1 and 2. In the range of pH 7—10.5, the semi-logarithmic plots of remaining reducing power *vs.* reaction time are almost linear, as shown in Fig. 1. The decrease of reducing power was apparently first order. The reducing power arises from SH-groups of GSH and cysteinylglycine (the decomposition product), so that the decrease corresponds to the total desulfurization of GSH and cysteinylglycine in the reaction solution. The total desulfurization can be regarded as a first-order process. In the range of pH 11—12, the semi-logarithmic plots are almost linear at the early stage of desulfurization, as shown in Fig. 2, but later the desulfurization rates decrease with reaction time. In these cases, the first-order rate constants were calculated based on the early stage. The time courses in the case of 0.10 and 0.15 M buffer concentration showed similar linearity. Below pH 7, the reducing power decrease was much less than the total decomposition of GSH. Thus, we concluded that the desulfurization of GSH aqueous solution could be analyzed as a pseudo-first order reaction in the range of pH 7—12.

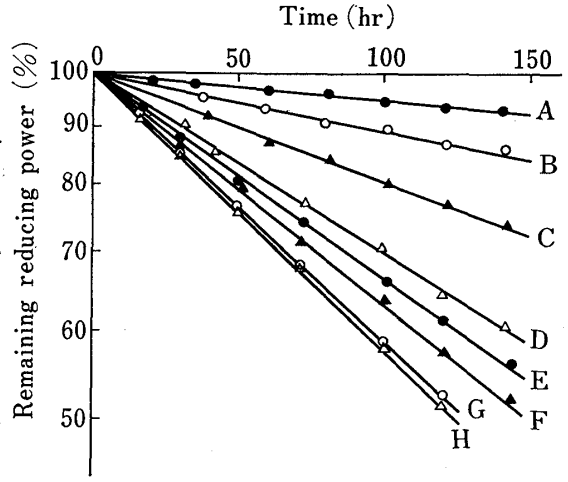

Fig. 1. Time Courses of Desulfurization in Aqueous Glutathione Solution (60°, $\mu=0.5$, buffer concentration; 0.05 M)

—●—A; pH 7.40, —○—B; pH 7.81, —▲—C; pH 8.43, —△—D; pH 8.91, —●—E; pH 9.37, —▲—F; pH 9.69, —○—G; pH 10.00, —△—H; pH 10.48.

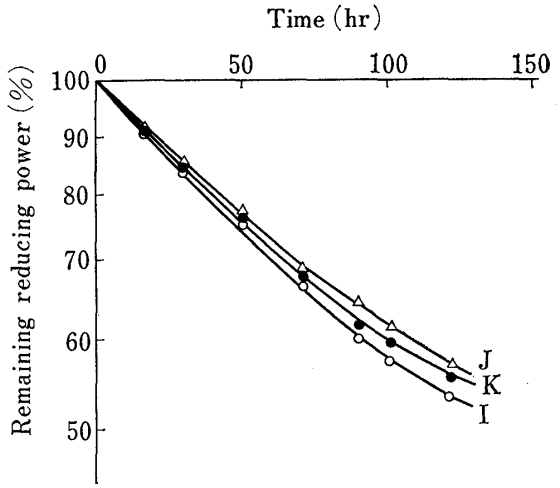

Fig. 2. Time Courses of Desulfurization in Aqueous Glutathione Solution (60°, $\mu=0.5$, buffer concentration; 0.05 M)

—○—I; pH 10.99, —△—J; pH 11.55, —●—K; pH 12.00.

Effect of Buffer Concentration on the Desulfurization Rate

Figure 3 shows the effect of buffer concentration on the desulfurization rate constants. In both phosphate buffer and borate buffer, the effect of buffer concentration was only slight. The buffer-free rate constants, k_{des}, at various pH values were calculated by extrapolation of the data shown in Fig. 3.

Primary Salt and Ethanol Effects

The time courses of desulfurization at ionic strengths of 0.02 and 0.30 (adjusted with potassium chloride) at pH 8.5 and 10.5 are shown in Fig. 4. Those at ionic strengths of 0.04, 0.07, and 0.10 were virtually the same as these. The time courses of desulfurization in 0 and 45% ethanol solution at pH 8.5 are shown in Fig. 5. Those for 10 and 30% ethanol solution were essentially the same as in the case without ethanol. We concluded that the

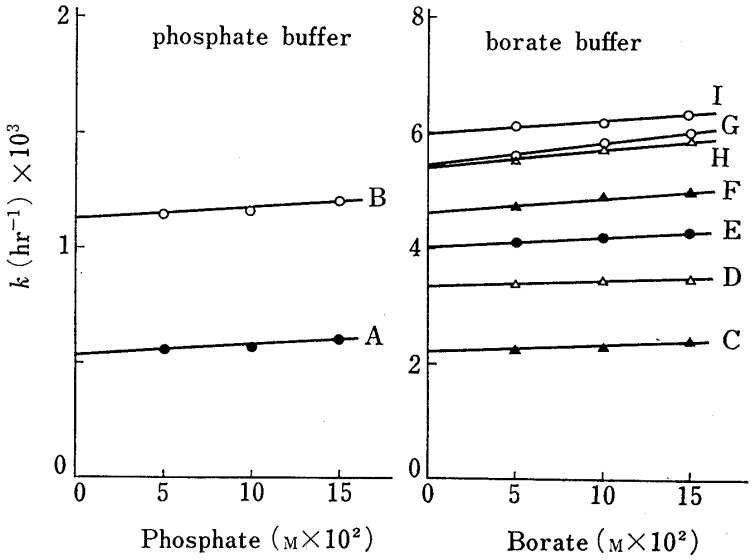

Fig. 3. Effect of Buffer Concentration on the Desulfurization Rate Constant of Glutathione Solution

Symbols, see legends to Fig. 1 and 2.

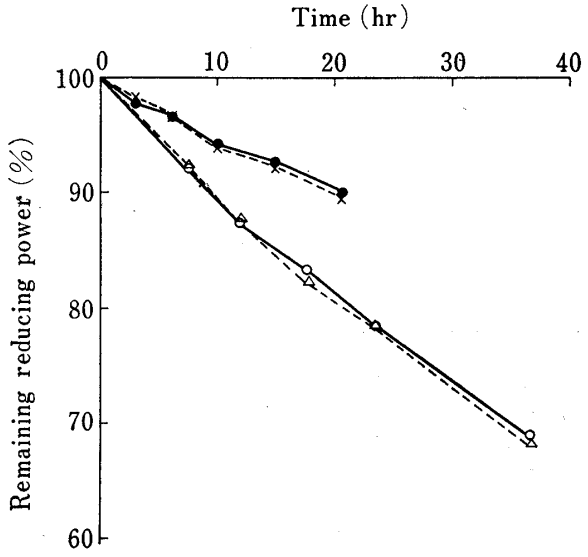

Fig. 4. Time Courses of Desulfurization of Unbuffered Glutathione Solution (60°, $\mu=0.02$ and 0.30)

—●—; pH 8.50, $\mu=0.02$, ---×---; pH 8.50, $\mu=0.30$,
—○—; pH 10.50, $\mu=0.02$, ---△---; pH 10.50, $\mu=0.30$.

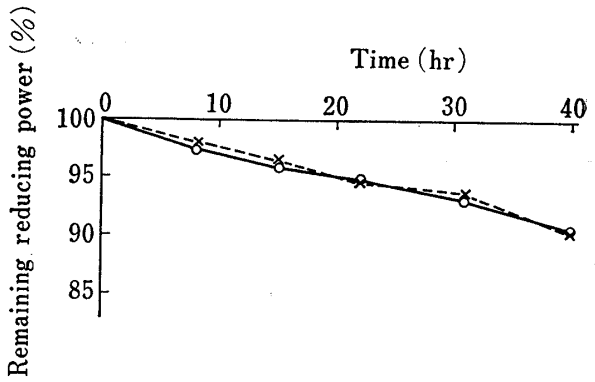

Fig. 5. Time Courses of Desulfurization of Glutathione in 0 and 45% Ethanol Solution at pH 8.55 at 60°

—○—; 0% ethanol, ---×---; 45% ethanol.

effects of ionic strength and ethanol (dielectric constant effect) on the desulfurization reaction were almost negligible.

pH Profile of the Desulfurization Rate Constant

The log k_{des}-pH profile (Fig. 6) was constructed from the buffer-free first-order rate constants of desulfurization and the pH values. The desulfurization in neutral solution takes place very slowly, but in the range of pH 7—9 the desulfurization rate increases as the pH rises, reaching a plateau in the pH 10 to 12 region.

Rate Constants of γ-Glutamyl Bond Cleavage and the pH Profile

GSH is decomposed mainly to pyroglutamic acid and cysteinylglycine in anaerobic aqueous solution, and above pH 7 desulfurization of GSH takes place simultaneously.[3)]

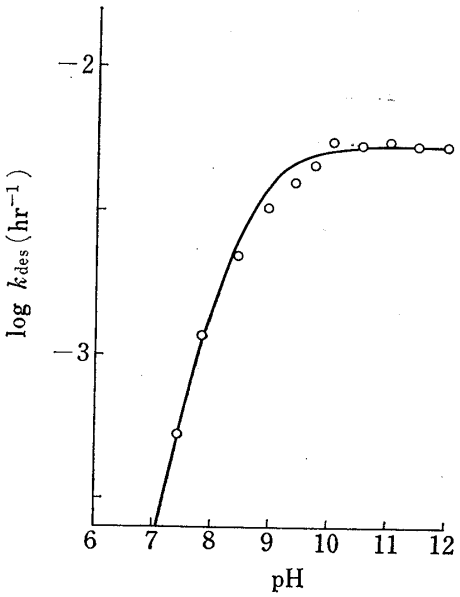

Fig. 6. Log (Desulfurization Rate Constant)-pH Profile (60°, $\mu=0.5$)

——; calculated from equation (22),
○; experimental data.

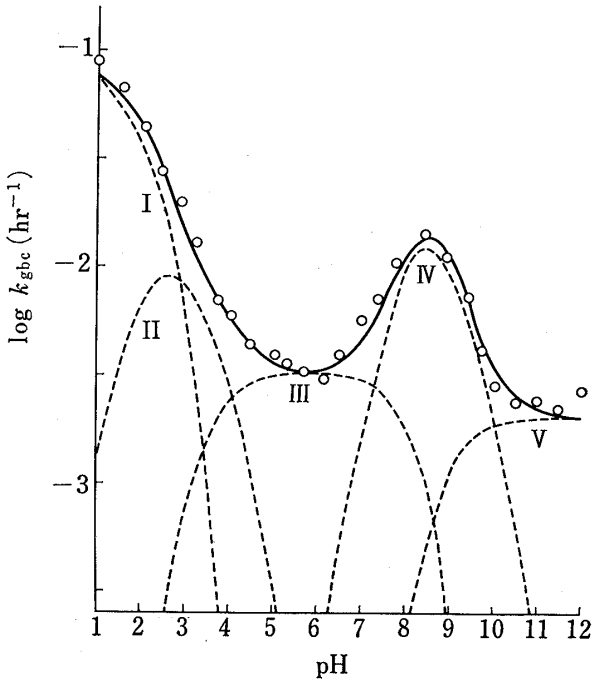

Fig. 7. log (γ-Glutamyl Bond Cleavage Rate Constant)-pH Profile (60°, $\mu=0.5$)

——; calculated from equation (20),
----; the five dotted-line segments I-V correspond to the five terms of equation (19),
○; experimental data.

Cysteinylglycine (a decomposition product), of course, undergoes desulfurization. The main decomposition reactions of GSH are shown in Chart 1.

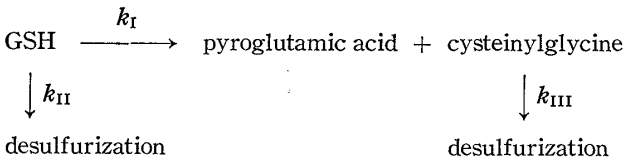

Chart 1

In Chart 1, k_I is the rate constant of cleavage of the γ-glutamyl bond of GSH, while k_{II} and k_{III} are the rate constants of desulfurization GSH and cysteinylglycine. A preliminary examination (see below) suggested that k_{II} was similar to k_{III}. GSH (5 g) was dissolved in 10 ml of hot water, poured in to ampoules, sealed under N_2 gas and heated for 20 hours at 70°. GSH was 89% decomposed and pyroglutamic acid and cystenylglycine were produced essentially quantitatively. From this solution, reaction solutions (pH 7.79 and pH 9.71) were prepared in the same way as for the experiments shown in Fig. 1. The time courses of desulfurization at 60° were similar to time courses B (pH 7.81) and F (pH 9.69) in Fig. 1. The results suggested that k_{II} was similar to k_{III}. Furthermore, the desulfurization was first order, so that despite the decrease of GSH and the formation of cysteinylglycine the desulfurization rate constant of the reaction solution was essentially constant at any reaction time. On the other hand, the rate constants of total decomposition of GSH were about 5.7×10^{-3} -1.7×10^{-2} $hour^{-1}$ in the range of pH 7—12 at 60°, and the rate constants of desulfurization were about 5×10^{-4} -5×10^{-3} $hour^{-1}$ as shown in Fig. 6. Now, the desulfurization rate constant of cysteinylglycine, k_{III}, is assumed to be equal to that of GSH, k_{II}. Accordingly, the desulfurization rate constant, k_{des}, calculated from the data on remaining reducing power

and reaction time can be taken as equal to both k_{II} and k_{III}. The total anaerobic decomposition rate constant, k_{tot}, is the sum of the cleavage rate constant of the γ-glutamyl bond, k_{gbc} (k_I in Chart 1), and the desulfurization rate constant, k_{des} (k_{II}), *i.e.*,

$$k_{gbc} = k_{tot} - k_{des} \quad (1)$$

Below pH 7, desulfurization was negligible compared to the total decomposition of GSH, so that k_{gbc} could be taken as equal to k_{tot}. The values of k_{tot} at various pHs were reported in the preceding paper.[1] The values of k_{gbc} were calculated from those values of k_{tot} and k_{des} in this study. The log k_{gbc}-pH profile is shown in Fig. 7; it is interesting that the profile is bell-shaped in the pH 6 to 10 region.

Effects of Temperature on the Cleavage of the γ-Glutamyl Bond and on Desulfurization

The temperature dependences of the cleavage of the γ-glutamyl bond and of desulfurization were estimated by determining k_{gbc} and k_{des} at 50°, 60°, 70° and 80°. Arrhenius plots of log k_{gbc} and k_{des} *vs.* 1/T were linear. The activation energies calculated from the slopes are shown in Table I. The values were about 19—21 kcal/mol.

Table I. Apparent Activation Energies of γ-Glutamyl Bond Cleavage and Desulfurization of Glutathione

	Apparent activation energy (kcal/mol)	
	γ-Glutamyl bond cleavage	Desulfurization
pH 2.84	18.9	
pH 5.56	20.9	
pH 8.43	21.4	21.1
pH 10.00	19.6	21.0

Relationship between the Ionic Form of GSH and the Rate Constant of Cleavage of the γ-Glutamyl Bond

GSH can exist in various ionized forms in aqueous solution, as shown in Chart 2. G_2 and G_4 were investigated as described for the total decomposition of GSH, where G_2 is the sum of G_{21} (HOOC–glu–cys–gly–COO$^-$) and G_{22} ($^-$OOC–glu–cys–gly–COOH), and G_4 is the sum of G_{41} and G_{42}.[1] The apparent dissociation constants, K_1, K_2, K_3 and K_4 at an ionic strength of 0.5 at 60° were $10^{-2.00}$, $10^{-3.51}$, $10^{-8.14}$ and $10^{-8.85}$.[1] The calculated mole fractions of each ionic species of GSH at arbitrary pH are shown in Table II.

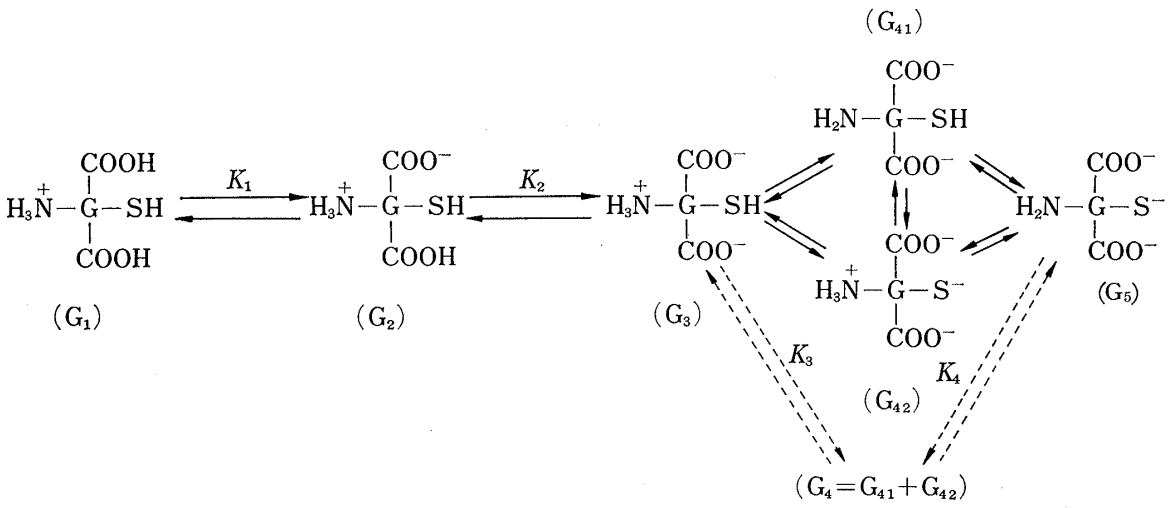

Chart 2

Table II. Mole Fraction of Ionic Species of Glutathione in Aqueous Solution

Ionic species of glutathione	Mole fraction	Symbol for mole fraction
G_1	$a_H^4/(A\times B)$	FG_1
G_2	$10^{-2}a_H^3/(A\times B)$	FG_2
G_3	$3.09\times10^{-6}a_H^2/(A\times B)$	FG_3
G_4	$3.24\times10^{-14}a_H/(A\times B)$	FG_4
G_5	$3.16\times10^{-23}/(A\times B)$	FG_5

a) $a_H=f_H\times[H^+]$ f_H; activity coefficient of hydrogen ions
b) $(A\times B)=(a_H^2+10^{-2.66}a_H+3.09^{-6})\ (a_H^2+7.24\times10^{-9}a^H+1.02\times10^{-17})$.

Given that the cleavage of the γ-glutamyl bond consists of the sum of spontaneous reactions and acid-base catalyzed reactions, and assuming that possible reactions are (a)—(m) in Chart 3, based on the pH profile in Fig. 7, the cleavage rate of the γ-glutamyl bond can be expressed by equation (2).

$$\begin{aligned}-k_{gbc}\cdot[G]_T = {} & k_1[G_1] + k_2[G_1]K_W/a_H + k_3[G_2] + k_4[G_2]\cdot a_H \\ & + k_5[G_2]\cdot K_W/a_H + k_6[G_3] + k_7[G_3]\cdot a_H \\ & + k_8[G_3]\cdot K_W/a_H + k_9[G_4] + k_{10}[G_4]\cdot a_H \\ & + k_{11}[G_4]\cdot K_W/a_H + k_{12}[G_5] + k_{13}[G_5]\cdot a_H \end{aligned} \quad (2)$$

$$[G]_T = [G_1] + [G_2] + [G_3] + [G_4] + [G_5] \quad (3)$$

K_W; ion product constant of water

On the other hand, equations (4)—(11) express the relationship between the dissociation constants and the concentration of ionic species at arbitrary pH.

$$[G_1]\cdot K_W/a_H = [G_2]\cdot K_W/K_1 \quad (4)$$

$$[G_2]\cdot a_H = [G_1]\cdot K_1 \quad (5)$$

$$[G_2]\cdot K_W/a_H = [G_3]\cdot K_W/K_2 \quad (6)$$

$$[G_3]\cdot a_H = [G_2]\cdot K_2 \quad (7)$$

$$[G_3]\cdot K_W/a_H = [G_4]\cdot K_W/K_3 \quad (8)$$

$$[G_4]\cdot a_H = [G_3]\cdot K_3 \quad (9)$$

$$[G_4]\cdot K_W/a_H = [G_5]\cdot K_W/K_4 \quad (10)$$

$$[G_5]\cdot a_H = [G_4]\cdot K_4 \quad (11)$$

Combination of equation (2) and equations (4)—(11) leads to equation (12).

$$\begin{aligned}-k_{gbc}[G]_T = {} & (k_1+k_4K_1)[G_1] \\ & + (k_2K_W/K_1+k_3+k_7K_2)[G_2] + (k_5K_W/K_2+k_6+k_{10}K_3)[G_3] \\ & + (k_8K_W/K_3+k_9+k_{13}K_4)[G_4] + (k_{11}K_W/K_4+k_{12})[G_5] \end{aligned} \quad (12)$$

Hence,

$$\begin{aligned}-k_{gbc} = {} & (k_1+k_4K_1)FG_1 + (k_2K_W/K_1+k_3+k_7K_2)FG_2 \\ & + (k_5K_W/K_2+k_6+k_{10}K_3)FG_3 + (k_8K_W/K_3+k_9+k_{13}K_4)FG_4 \\ & + (k_{11}K_W/K_4+k_{12})FG_5 \end{aligned} \quad (13)$$

Parameters X_1, X_2, X_3, X_4 and X_5 are defined by equations (14)—(18)

$$X_1 = k_1 + k_4K_1 \quad (14)$$

$$X_2 = k_2K_W/K_1 + k_3 + k_7K_2 \quad (15)$$

$$X_3 = k_5K_W/K_2 + k_6 + k_{10}K_3 \quad (16)$$

$$X_4 = k_8K_W/K_3 + k_9 + k_{13}K_4 \quad (17)$$

$$X_5 = k_{11}K_W/K_4 + k_{12} \quad (18)$$

Combining equation (13) with equations (14)—(18), the rate constant, k_{gbc}, can be expressed as follows.

$$k_{gbc} = X_1 \cdot FG_1 + X_2 \cdot FG_2 + X_3 \cdot FG_3 + X_4 \cdot FG_4 + X_5 \cdot FG_5 \quad (19)$$

Thus, k_{gbc} at an arbitrary pH is expressed as a function of a_H. The parameters X_1—X_5 were calculated by the least-squares method by fitting equation (19) to the experimental rate constants of cleavage of the γ-glutamyl bond at various pHs. The values obtained are shown in Table III. Equation (20) is obtained by substituting the values in Tables II and III into equation (19).

$G_1 \xrightarrow{k_1}$ products	reaction (a)[a]	$G_3 + OH^- \xrightarrow{k_8}$ products	reaction (h)
$G_2 + OH^- \xrightarrow{k_2}$ products	reaction (b)[b]	$G_4 \xrightarrow{k_9}$ products	reaction (i)
$G_2 \xrightarrow{k_3}$ products	reaction (c)	$G_4 + H^+ \xrightarrow{k_{10}}$ products	reaction (j)
$G_2 + H^+ \xrightarrow{k_4}$ products	reaction (d)	$G_4 + OH^- \xrightarrow{k_{11}}$ products	reaction (k)
$G_2 + OH^- \xrightarrow{k_5}$ products	reaction (e)	$G_5 \xrightarrow{k_{12}}$ products	reaction (l)
$G_3 \xrightarrow{k_6}$ products	reaction (f)	$G_5 + H^+ \xrightarrow{k_{13}}$ products	reaction (m)
$G_3 + H^+ \xrightarrow{k_7}$ products	reaction (g)		

a) This represents the cleavage reaction of the γ-glutamyl bond of G_1 proceeding spontaneously or with catalysis by water.
b) This represents the cleavage reaction of the γ-glutamyl bond of G_2 proceeding with catalysis by OH^-.

Chart 3

TABLE III. Obtained Parameters for Equation(19)

X_1	X_2	X_3	X_4	X_5
8.21×10^{-2}	1.20×10^{-2}	3.38×10^{-3}	2.29×10^{-2}	$2.04\times^{-3}$

(unit; hr^{-1})

$$k_{gbc} = (8.21\times10^{-2}a_H^4 + 1.20\times10^{-4}a_H^3 + 1.04\times10^{-8}a_H^2 + 5.13\times10^{-16}a_H + 6.44\times10^{-26})/(a_H^2 + 10^{-2}a_H + 3.09\times10^{-6})(a_H^2 + 7.24\times10^{-9}a_H + 1.02\times10^{-17}) \quad (20)$$

(unit: hr^{-1})

Equation (20) expresses the cleavage constant of the γ-glutamyl bond as a function of hydrogen ion activitity at arbitrary pH. The solid line in Fig. 7 is the line calculated using equation (20), and it agrees well with the experimental values. This calculated curve can be taken as the sum of curves I, II, III, IV and V (dotted lines), corresponding to G_1, G_2, G_3, G_4 and G_5. In the pH range of 1—4.5, the curve calculated with equation (20) is considered to be the sum of curves I and II principally. Because the concentration of hydroxide ions is extremely small, reaction (b) in Chart 3 may not contribute much to the cleavage reaction. In this pH range, the dominant reactions are, therefore, the spontaneous or water-catalyzed cleavage of G_1 and G_2, and hydrogen ion-catalyzed cleavage of G_2 and G_3. In the pH range 4.5—6.5, the mole fractions of both G_2 and G_4 are very small, so the dominant reaction (curve III) may be the spontaneous or water-catalyzed cleavage of the γ-glutamyl bond of G_3. This is supported by the experimental findings that ionic strength and dielectric constant do not influence the cleavage rate. Although hydroxide ion-catalyzed cleavage of G_3 and hydrogen ion-catalyzed cleavage of G_5 are possible reactions in the pH

range of 7.5—10, the effect of ionic strength on the rate constant is negligible and ethanol addition also has only a small effect. Reaction (h) and reaction (m) in Chart 3 may, therefore, not contribute much to the cleavage reaction, and the dominant reaction (curve IV) may be mainly the spontaneous or water-catalyzed cleavage of the γ-glutamyl bond of G_4. It is interesting that the log k_{gbc}-pH profile is bell-shaped in the pH 7.5 to 10 region and that the γ-glutamyl bond of G_4 is cleaved much more rapidly than those of G_3 and G_5. Calvin[6] suggested that GSH may form ring conformations: namely, hydroxythiazoline form, thiazoline form, pyrrolidoneimonium form, and hydroxypyrrolidone form. Isherwood[7] also assumed the formation of ring conformations. Peters *et al.*[8] reported that the intramolecular proton transfer between G_{41} ($-NH_2$, $-SH$) and G_{42} ($-NH_3^+$, $-S^-$) in Chart 2 took place directly in aqueous solution. Pyroglutamic acid, which is one product of cleavage of the γ-glutamyl bond of GSH, also has a ring conformation. Thus, it seems likely that the NH_2 group and SH group of GSH may exist in the vicinity of the γ-glutamyl bond in aqueous solution, as shown in Chart 4. As mentioned above, the spontaneous or water-catalyzed cleavage of the γ-glutamyl bond of G_4 is much more rapid than those of both G_3 and G_5. It is thought that the dissociation of NH_2 and SH governs the cleavage rate, so that the cleavage of the γ-glutamyl bond is accelerated, at least in the range of pH 7.5—10, by a kind of intramolecular catalysis. This is consistent with the finding that the cysteinyl bond of GSH is much more stable in aqueous solution than the γ-glutamyl bond.

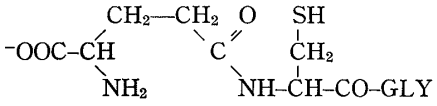

Chart 4

Relationship between the Ionization State of GSH and the Rate Constant of Desulfurization

The desulfurization rate constant, k_{des}, is negligible below pH 7. k_{des} was assumed to be given by equation (21), and the parameters X_4' and X_5' were calculated using the least-squares method by fitting equation (21) to the experimental rate constants of desulfurization at various pHs. The values obtained are shown in Table IV.

$$k_{des} = X_4' \cdot FG_4 + X_5' \cdot FG_5 \quad (21)$$

Equation (22) was obtained by substituting the values in Table IV into equation (21).

$$k_{des} = (6.43 \times 10^{-17} a_H + 1.62 \times 10^{-25})/(a_H^2 + 10^{-2} a_H + 3.09 \times 10^{-6})(a_H^2 + 7.24 \times 10^{-9} a_H + 1.02 \times 10^{-17}) \quad (22)$$

(unit : hr^{-1})

Equation (22) expresses the rate constant of desulfurization as a function of hydrogen ion activity (a_H) at arbitrary pH. The solid line in Fig. 6. is the curve calculated from equation (22); it coincides reasonably well with the experimental values.

Table IV. Obtained Parameters for Equation (21)

X_4'	X_5'
2.87×10^{-3}	5.13×10^{-3}

(unit; hr^{-1})

6) M. Calvin, "Glutathione," Edited by S. Colowick, Academic Press Inc., New York, 1954, p. 25.
7) F.A. Isherwood, "Glutathione," Edited by E.M. Crook, Cambridge University Press, 1959, p. 14.
8) F. Peters and G. Maass, "Glutathione," Edited by Flohe *et al.*, Georg Thieme Publishers, Stuttgart, 1974, p. 15.