Coenzyme Models. 25. Facile Oxidation of Hemithiol Acetals by Flavin. Supporting Evidence for the Enediol Mechanism of Glyoxalase I[1]

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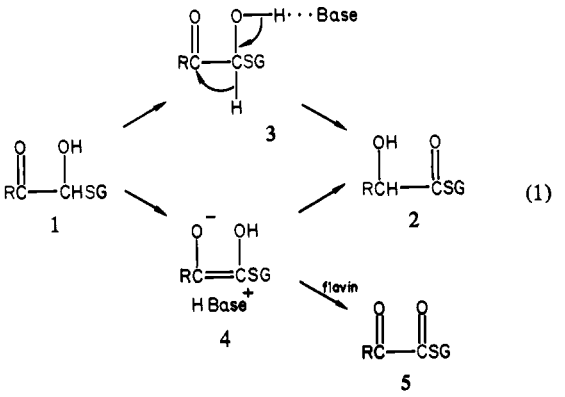
Abstract: The rearrangement of hemithiol acetals (**1**) to α-hydroxythiol esters (**2**) was inhibited almost completely on the addition of 3-methyltetra-*O*-acetylriboflavin (MeFl), and corresponding α-keto acids were produced in good yields. Since (i) flavin is able to trap transient enediol and intermediates oxidatively and (ii) flavin does not serve as an efficient oxidant for intermediates including the 1,2-hydride shift mechanism (Cannizzaro reaction), the finding supports, in agreement with the results of Hall et al., the idea that the rearrangement (and also the enzymic catalysis by glyoxalase I) occurs via the enediol intermediate. The kinetic measurements, which were carried out conveniently by following the disappearance of the absorption band of MeFl, established that (i) the oxidation by MeFl is zero order in MeFl and first order in thiols and glyoxals, (ii) the rate constant for (dimethylamino)ethanethiol is greater by a factor of about 50 than that for 2-mercaptoethanol, (iii) the k_H/k_D values observed for C_6H_5COCHO vs. C_6H_5COCDO are 5–12, and (iv) the K_a value of the hemithiol acetal of methylglyoxal as carbon acid is estimated on the basis of linear free-energy relationships to be $10^{-11.3}$. The results show that the reaction sequence consists of the rate-limiting deprotonation from hemithiol acetal to form the enediol intermediate followed by the rapid oxidation by MeFl. It is proposed that the hemithiol acetal is classified as a carbon acid and the dissociation is facilitated owing to the electron-withdrawing nature of the acyl group and the carbanion-stabilizing effect of the sulfur atom.

The mechanism by which the glyoxalase enzyme system catalyzes the conversion of glyoxals to corresponding α-hydroxy acids is a controversial problem. The enzyme requires glutathione (GSH) as cofactor[2] (eq 1). Mechanistically, the most interesting



(1)

step in the reaction sequence is the rearrangement of hemithiol acetal (**1**) to α-hydroxythiol ester (**2**) that is catalyzed by glyoxalase I. The reaction sequence is analogous to the Cannizzaro reaction, which has been fairly convincingly established to employ the 1,2-hydride shift mechanism.[3],[4a] The mechanism of rearrangement of **1** to **2** still involves a controversial problem. The 1,2-hydride shift mechanism (**3**) that was originally proposed by Franzen[5] and later supported by Rose[6] has been generally accepted. In contrast to their proposal, Hall et al.[4] recently presented evidence that the rearrangement occurs via the enediol intermediate (**4**) (originally proposed by Racker)[7] rather than via the 1,2-hydride shift. Apparently, the two mechanisms can be distinguished by detecting the incorporation (or lack of incorporation) of solvent protons into the product. Indeed, Franzen[5a] and Rose[6] proposed the 1,2-hydride shift mechanism for glyoxalase I on the basis of the lack of detection of deuterium (3% maximum incorporation) or low incorporation of tritium (less than 4%) in the lactic acid product. On the other hand, the investigation by Hall et al.[4] provided evidence that considerable amounts of solvent protons are incorporated into the product, which is in agreement with the enediol mechanism. Hall et al.[4] rationalized the disparity in terms of the possibility of a fast proton-transfer mechanism via an enediol taking place in a highly protected active site of glyoxalase I.

The foregoing discussions have been based only on the solvent incorporation technique. Recently, Douglas and Nadvi[8] reported that the glyoxalase I catalysis is efficiently inhibited by compounds including the 1,2-enediol structure unit. We considered that the application of quite different techniques would provide further convincing evidence. We thus applied the "Flavin-trapping technique"[9] to the glyoxalase I model system. It has been established that a considerable number of flavoproteins employ the carbanionic intermediates in the course of the enzymic oxidations.[10,11] This is applicable to nonenzymatic oxidation of transient carbanion intermediates (flavin-trapping).[9] In most cases, the reaction is zero order in flavin,[9,12] indicating that the oxidative trapping by flavin is very fast. On the other hand, flavin does not serve as an efficient hydride acceptor.[1,13] This indicates that, if the reaction proceeds via the enediol intermediate (**4**), added flavin would inhibit the rearrangement and divert the reaction sequence to the oxidation reaction resulting in α-ketothiol ester (**5**). In this paper, we wish to report that the rearrangement of **1** to **2** is almost completely inhibited on the addition of flavin. The finding confirms the results of Hall et al.[4] that **4** is an obligatory

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(3) Hine, J.; Koser, G. *J. Org. Chem.* **1971**, *36*, 3591 and references cited therein.
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(5) (a) Franzen, V. *Chem. Ber.* **1956**, *89*, 1020. (b) *Ibid.* **1955**, *88*, 1361. (c) *Ibid.* **1957**, *90*, 623.
(6) Rose, I. A. *Biochim. Biophys. Acta* **1957**, *25*, 214.
(7) Racker, E. *J. Biol. Chem.* **1951**, *190*, 685.
(8) Douglas, K.; Nadvi, I. N. *FEBS Lett.* **1979**, *106*, 393.
(9) (a) Shinkai, S.; Ide, T.; Manabe, O. *Chem. Lett.* **1978**, 583. (b) Shinkai, S.; Yamashita, T.; Manabe, O. *J. Chem. Soc., Chem. Commun.* **1979**, 301. (c) Shinkai, S.; Yamashita, T.; Kusano, Y.; Ide, T.; Manabe, O. *J. Am. Chem. Soc.* **1980**, *102*, 2335.
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(13) Hemmerich, P.; Massey, V.; Fenner, H. *FEBS Lett.* **1977**, *84*, 5.

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Table I. Product Analysis for the Flavin Trapping of Hemithiol Acetal from Phenylglyoxal (Ar = C_6H_5-)[a]

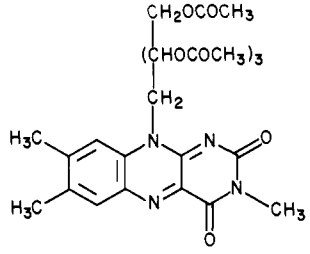
thiol (mM)	MeFl, mM	% yield ArCH(OH)CO_2H	% yield $ArCOCO_2H$	% yield $ArCO_2H$
$HO(CH_2)_2SH$ (130)	0	61	3.3	0
$HO(CH_2)_2SH$ (130)	13	0	76–81	5.6
$Me_2N(CH_2)_2SH$ (130)	13	0	55	0.3
glutathione (65)	13	0	13	3.0
C_6H_5SH (130)	13	0	6.1	1.7

[a] [Phenylglyoxal] = 1.30×10^{-2} M, 30 °C, pH 9.35 with borate buffer (0.1 M), 25% v/v of ethanol, 5 h in the dark.

intermediate in the rearrangement from **1** to **2**.

Results and Discussion

Flavin Trapping of Hemithiol Acetal. In the first place, we examined whether flavin is reduced by the reaction systems involving intermolecular or intramolecular hydride transfer. We used 3-methyltetra-*O*-acetylriboflavin (MeFl).[13] It was found



MeFl

that under strictly anaerobic (N_2) conditions, the flavin is not reduced either by the formaldehyde (0.1 M) plus NaOH (0.05 M) system or by the phenylglyoxal (0.01 M) plus NaOH (0.05 M) system. The spectral change observed was in agreement with that of the simple hydrolytic decomposition, and introduction of oxygen into the reaction cell did not regenerate the absorption spectrum characteristic of the oxidized flavin. On the basis of high-pressure LC analysis, we found that mandelic acid is produced almost quantitatively from the reaction of phenylglyoxal and NaOH (85% in the absence of MeFl and 82% in the presence of 1.0 mM MeFl). The result clearly indicates that flavin is hardly reduced by hydride reagents.

Intermediate **4** may be produced from **2**, which is formed by the rearrangement via **3**. Hence, one should take another reaction route into consideration: **1** → **3** → **2** → **4** → **5**. To exclude the possibility, we subjected *S*-ethyl thiomandelate (analogue of **2**) to flavin trapping. In a borate buffer (pH 9.35) solution containing MeFl (17 mM), *S*-ethyl thiomandelate (17 mM) was converted to mandelic acid almost quantitatively (84–95% yield), and benzoylformic acid was not detected by the high-pressure LC analysis. The result implicates that the hydrolysis of the thioester is much faster than the deprotonation to yield **4**. The conclusion was further corroborated by a kinetic study. When an ethanolic solution of *S*-ethyl thiomandelate (5.00×10^{-4} M) was mixed with the borate buffer solution of MeFl (5.00×10^{-5} M) in an anaerobic Thunberg cuvette, we could not observe the reduction of MeFl. It is clear, therefore, that if the reduction of MeFl occurs, the active species must be given directly from hemithiol acetal (**1**).

The rearrangement reaction of phenylglyoxal was carried out anaerobically (N_2) in an aqueous thiol solution. The results of the product analysis are summarized in Table I. It is seen from Table I that the main product in the absence of MeFl is mandelic acid, while the product distribution is markedly changed on the addition of MeFl. The rearrangement to mandelic acid is inhibited almost completely, and benzoylformic acid becomes the main product. The formation of benzoic acid in low yields is attributed to further oxidation of benzoylformic acid. The reaction catalyzed by 2-mercaptoethanol and (dimethylamino)ethanethiol afforded benzoylformic acid in relatively high yields, while the yields were low in GSH and thiophenol solutions.

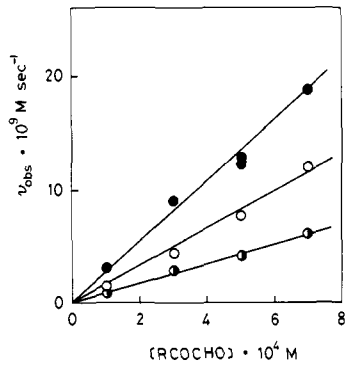


Figure 1. Flavin-trapping rate vs. concentration of glyoxal at 30 °C, pH 9.50 with 0.02 M borate buffer, μ = 0.1 with KCl, 25% v/v of ethanol, [2-mercaptoethanol] = 2.00×10^{-3} M, and [MeFl] = 5.00×10^{-5} M. Substrate: (●) 4-chlorophenylglyoxal, (○) phenylglyoxal, (◑) methylglyoxal.

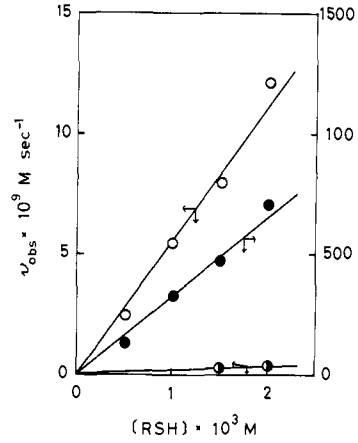


Figure 2. Flavin-trapping rate vs. concentration of thiols at 30 °C, pH 9.50 with 0.02 M borate buffer, μ = 0.1 with KCl, 25% v/v of ethanol, [4-chlorophenylglyoxal] = 5.00×10^{-4} M, [MeFl] = 5.00×10^{-5} M. Thiol: (●) (dimethylamino)ethanethiol, (○) 2-mercaptoethanol, (◑) GSH.

Reaction Order of Glyoxals and Thiols. Kinetic measurements of this section were performed at 30 °C, pH 9.5 under anaerobic conditions (for the detail see Experimental Section). The reaction was monitored spectrophotometrically by following the disappearance of MeFl at 448 nm. It is known that flavin is slowly reduced by aliphatic thiols (but not by aromatic thiols).[14] The present study also showed that 2-mercaptoethanol, (dimethylamino)ethanethiol, and GSH are able to reduce MeFl according to first-order kinetics with respect to MeFl. On the other hand, the disappearance of MeFl in the presence of both glyoxal and thiol was zero order (up to 90% reaction for 4-chlorophenylglyoxal and 65% reaction for phenylglyoxal). When compared by the initial velocities, the rate constants for the reduction by thiol were smaller by about 2 orders of magnitude than those by thiol plus glyoxal. For example, the initial velocity for the reduction of MeFl (5.00×10^{-5} M) by 2-mercaptoethanol (2.00×10^{-3} M) at pH 9.5 was 2.0×10^{-10} M s^{-1}. When 4-chlorophenylglyoxal (5.00×10^{-4} M) was added, the velocity was enhanced up to 1.31×10^{-8} M s^{-1}. The relative contribution of the thiol reduction is only 1.5%. The concentration of free thiols should be further reduced in the presence of glyoxals owing to the formation of hemithiol acetals. Hence, the contribution of the thiol reduction was neglected in the following discussion.

As shown in Figure 1 and 2, the rate of the flavin trapping was first order in thiols and glyoxals. The rate equation can be thus expressed by eq 2. The kinetic situation is in agreement with

$$v_{obsd} = k_{2,app}[\text{thiol}]^{1.0}[\text{glyoxal}]^{1.0}[\text{MeFl}]^0 \quad (2)$$

(14) (a) Loechler, E. L.; Hollocher, T. C. *J. Am. Chem. Soc.* **1975**, *97*, 3236. (b) Yokoe, I.; Bruice, T. C. *Ibid.* **1975**, *97*, 450.

Table II. Apparent Second-Order Rate Constants ($k_{2,app} \times 10^3$ M^{-1} s^{-1}) for the Flavin Trapping of Hemithiol Acetals

thiol	4-Cl-C_6H_4CHO	C_6H_5CHO	CH_3COCHO
$Me_2N(CH_2)_2SH$	640[a]		
$HO(CH_2)_2SH$	11.4,[a] 13.8[b]	8.68[b]	4.38[b]
glutathione	0.31[a]		
C_6H_5SH	0,[a] 0[b]		

[a] Determined from Figure 1. [b] Determined from Figure 2.

Table III. Apparent Second-Order Rate Constants ($\times 10^3$ M^{-1} s^{-1}) for Phenylglyoxal and [1-^{2}H]Phenylglyoxal[a]

thiol	pH	C_6H_5-COCHO	C_6H_5-COCDO	k_H/k_D
$HO(CH_2)_2SH$	9.90 (borate)	6.42	1.15	5.58
	8.60 (phosphate)	1.16	0.22	5.27
$Me_2N(CH_2)_2SH$	9.90 (borate)	318	26.4	12.0
	8.60 (phosphate)	458	39.2	11.7

[a] 30 °C, [phenylglyoxal] = 1.30×10^{-2} M, [MeFl] = 5.00×10^{-5} M, [thiol] = 2.00×10^{-3} M.

the cyanide-assisted oxidation of aldehydes and α-keto acids by flavin.[9] On the basis of the above finding, we calculated apparent second-order rate constants ($k_{2,app}$) and summarized them in Table II.

Examination of Table II reveals that (i) the reactivity of glyoxals is in order 4-chlorophenylglyoxal > phenylglyoxal > methylglyoxal, (ii) (dimethylamino)ethanethiol acts as 46–56 times more efficient catalyst than 2-mercaptoethanol, (iii) GSH which glyoxalase I requires as a cofactor is classified as a less effective catalyst in the model system, and (iv) thiophenol is totally ineffective. The high catalytic efficiency of (dimethylamino)ethanethiol is accounted for by the intramolecular general-base catalysis of the dimethylamino group.[5] No catalytic efficiency of thiophenol, which is compatible with the fact that the thiophenol-catalyzed system gives only 6.1% of benzoylformic acid (Table I), is attributable to the low nucleophilicity of thiophenol. The low catalytic efficiency of GSH was an unexpected result. The detailed examination of the spectral change indicated that only the GSH-catalyzed system affords a new absorption band at around 280 nm. The band can be attributed to the Schiff base.[15] Hence, the suppression of the GSH activity is accounted for by the nonproductive imine formation between the terminal amino group of GSH and glyoxal.

Deuterium Isotope Effect. As the rearrangement of **1** to **2** involves a proton transfer, it is of interest to measure the isotope effect on this reaction by substituting deuterium for hydrogen at the C-1 position of glyoxals. We synthesized [1-^{2}H]phenylglyoxal and obtained the kinetic isotope effect shown in Table III. Deuterium isotope effects for the glyoxalase I-catalyzed system and its model system have been reported.[4a,16,17] In the reaction catalyzed by yeast glyoxalase I (25 °C, pH 7.0), the magnitudes of the deuterium isotope effect ($V_{max,H}/V_{max,D}$) for methylglyoxal and phenylglyoxal were 2.9 and 3.2,[16] respectively, and varied widely depending on the metal ion employed.[17] In the model system with GSH as catalyst (30 °C, pH 7.0), the general-base (HPO_4^{2-})-catalyzed pathway gave rise to a kinetic isotope effect of 3.3 for methylglyoxal and 2.3 for phenylglyoxal.[4a] The k_H/k_D values determined by the falvin-trapping method with 2-mercaptoethanol as catalyst (Table III) were somewhat greater than the previously reported values.[4a,16] This is due probably to the difference in the reaction conditions. Srinivasan and Stewart[18] have reported that the kinetic isotope effect in the general-base-catalyzed deprotonation is a function of the basicity of the reagent — the lower the basicity of the reagent, the smaller the isotope effect. The rate constants determined by Hall et al.[4a] at pH 7.0 reflect the HPO_4^{2-} catalysis, whereas the rate constants in Table III were determined at relatively higher pH region (8.6–9.9) and reflect mostly, as seen in Figure 4, the catalysis by hydroxide ion. This difference may cause the difference in the magnitude of the kinetic isotope effects. In either case, the magnitude becomes evidence for the primary kinetic isotope effect, indicating that the proton transfer on C-1 is predominantly involved in the rate-determining step.

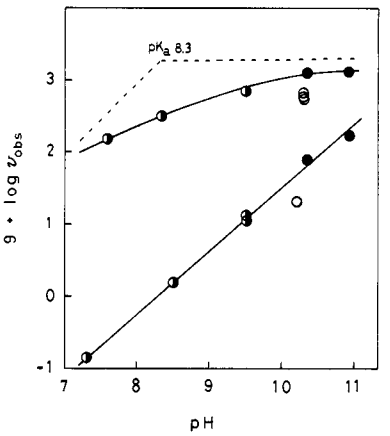


Figure 3. pH dependence for flavin trapping of hemithiol acetal of 4-chlorophenylglyoxal (5.00×10^{-4} M) at 30 °C, μ = 0.1 with KCl, 25% v/v of ethanol, [MeFl] = 5.00×10^{-5} M. Buffer (0.02 M): (●) carbonate, (○) borate, (◑) phosphate. Upper line is (dimethylamino)ethanethiol; lower line is 2-mercaptoethanol (2.00×10^{-3} M).

Interestingly, the magnitude of the kinetic isotope effect for (dimethylamino)ethanethiol was about 2 times greater than that for 2-mercaptoethanol (Table III). It is not clear, however, why the intramolecular base catalysis provided such a large isotope effect. It is known that when the sterically hindered base is used as a catalyst for the base-catalyzed deprotonation, the abnormally large isotope effect is observed.[19] This effect is attributed to the compressional energies of the atoms which provide the steric hindrance.[20] The intramolecular base catalysis may require such a compressional energy on the formation of the cyclic transition state.

pH Dependence. Logarithms of the flavin-trapping rates are plotted as a function of pH in Figure 3. The kinetic examination was performed at pH region lower than 11, because the rate of the Cannizzaro reaction becomes nonnegligible of significance at higher pH region. The pH dependence was thus examined between pH 7 and 11.

The rate of the 2-mercaptoethanol-mediated flavin trapping increases linearly with slope 0.88. Taking it into account that the increasing fraction of glyoxal is hydrated at high pH region,[21] one can conclude that the 2-mercaptoethanol-mediated flavin trapping is catalyzed by OH^-. The pH–rate profile for the (dimethylamino)ethanethiol-mediated system reflects the catalysis of both OH^- and the intramolecular dimethylamino group. The apparent pK_a at around 8.4 (Figure 3) is ascribable to the acid dissociation of the dimethylamino group. Since the nucleophilicity of the thiol group of (dimethylamino)ethanethiol is similar to that of 2-mercaptoethanol,[22] one may assume that the concentrations of two hemithiol acetals are almost identical. The contribution of the intramolecular dimethylamino group is thus evaluated from Figure 3 directly: that is, the catalytic efficiency amounts to 10^3-fold at pH 7.0 and 10^2-fold at pH 9.0.

(15) (a) Jencks, W. P. *J. Am. Chem. Soc.* **1959**, *81*, 475. (b) Matsuo, Y. *Ibid.* **1957**, *79*, 2061. (c) Matsushima, Y.; Martell, A. E. *Ibid.* **1967**, *89*, 1322.
(16) Vander Jagt, D. L.; Han, L.-P. B. *Biochemistry* **1973**, *12*, 5161.
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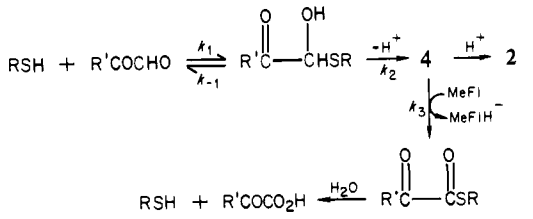
(22) (a) Whitaker, J. R. *J. Am. Chem. Soc.* **1962**, *84*, 1900. (b) Ogilvie, J. W.; Tildon, J. T.; Strauch, B. S. *Biochemistry* **1964**, *3*, 754. (c) Whitesides, G. M.; Lilburn, J. E.; Szajewski, R. P. *J. Org. Chem.* **1977**, *42*, 332.

Scheme I



As indicated in the legend for Figure 3, phosphate and carbonate buffers provide smooth pH–rate profiles. On the other hand, the data obtained in borate buffer solutions were smaller than those in other buffer solutions. The phenomenon was reproducible. The deviation is ascribed to the increase in the hydrated fraction of glyoxal in the borate buffer solutions. It is known that borate species can form complexes with diols.[23] Hydrated species of hemithiol acetals exactly includes the diol structure. In fact, Okuyama[24] noticed that borate buffer catalyzes the hydration of hemithiol acetals.

Reaction Scheme for the Glyoxalase I Model Reaction. On the basis of the foregoing results, we can not depict the reaction scheme (Scheme I) for the glyoxalase I model reaction. Since (i) the observed rate is zero order in MeFl and (ii) the large primary isotope effect is observed for [1-^{2}H]phenylglyoxal, the deprotonation from **1** (i.e., k_2) is assigned to the rate-limiting step and the subsequent flavin oxidation is a rapid, trapping step. The conclusion is also supported by the substituent effect. It is assumed that the electron-withdrawing substituent would facilitate the deprotonation from **1** (k_2) due to the enhanced acidity of the C-1 hydrogen but would suppress the oxidation by MeFl (k_3) because of the reduced basicity of **4**. The order of the rate constants 4-chlorophenylglyoxal > phenylglyoxal > methylglyoxal is clearly compatible with the rate-limiting deprotonation. The high catalytic efficiency observed for (dimethylamino)ethanethiol has been attributed to the intramolecular general-base catalysis of the dimethylamino group[5] and has frequently been cited as a typical example of enzyme-like bifunctional catalysis. However, both the NMR studies by Hall et al.[4a] and the present flavin-trapping data reveal that the amine base catalyzes the deprotonation from 2-C–H but not from 2-O–H as proposed by Franzen.[5]

The rate equation for Scheme I is expressed by eq 3, where K

$$v_{obsd} = \frac{k_2K[\mathrm{RSH}][\mathrm{R'COCHO}]}{1 + K[\mathrm{RSH}]} \qquad (3)$$

$= k_1/k_{-1}$. Since v_{obsd} was first order in RSH and R'COCHO, $1 \gg K$[RSH] is assumed under the present reaction conditions. According to the study of Kanchuger and Byers,[25] the K value for the association of methylglyoxal and GSH is about 0.3 M^{-1}. The K[RSH] values under the present experimental conditions are (1–6) × 10^{-3}, which are sufficiently smaller than unity. Hence, eq 3 can be rewritten as eq 4. Therefore, $k_{2,app}$ in eq 2 is equivalent to k_2K in eq 4.

$$v_{obsd} = k_2K[\mathrm{RSH}][\mathrm{R'COCHO}] \qquad (4)$$

Since the deprotonation from carbon acids is general base catalyzed,[26] the k_2 involves the base-catalyzed term. The K may be also affected by the dissociation of RSH. If so, the slope of the pH–rate profile (Figure 3) would be +2 at pH < p$K_{a,RSH}$ and +1 at pH > p$K_{a,RSH}$. In fact, however, Figure 3 indicates that the v_{obsd} is simply proportional to the concentration of OH$^-$ at pH 7–11. The p$K_{a,RSH}$ of 2-mercaptoethanol is 9.32.[22] It is presumed, therefore, that only the k_2 is a function of the base-catalyzed term.

Acidity of 1 as Carbon Acid. Hall et al.[4a] have found that the pseudo-first-order rate constant increases linearly with HPO_4^{2-} concentration, indicating the proton transfer to be buffer catalyzed. In order to obtain further insight into the mechanism, we carried out the buffer dilution study for the flavin oxidation of the 2-mercaptoethanol plus methylglyoxal system. The plots of $k_{2,app}$ vs. [buffer]$_T$ (total concentration of buffer) are shown in Figure 4. Since the deprotonation is catalyzed by buffer and OH$^-$, the $k_{2,app}$ is given by eq 5, where k_{gb}' and k_{OH} are second-order rate constants for the buffer catalysis and the OH$^-$ catalysis, respectively. Hence, the slope and the intercept in Figure 4 correspond to $k_{gb}'K$ and $k_{OH}K$[OH$^-$], respectively. The slopes and the intercepts were calculated by least-squares computation. From the intercepts the k_{OH} values were obtained (K = 0.3 M^{-1}).[25] Since the true buffer catalysis is associated with the dissociated fraction (α), true second-order rate constants for the general-base catalysis (k_{gb}) are equal to k_{gb}'/α. The k_{OH} and k_{gb} thus determined are summarized in Table IV.

$$k_{2,app} = k_2K = (k_{gb}'[\mathrm{buffer}]_T + k_{OH}[\mathrm{OH}^-])K \qquad (5)$$

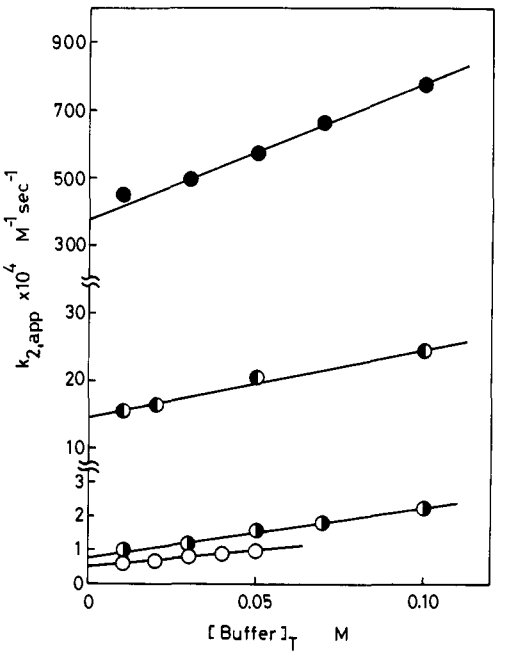


Figure 4. Buffer dilution with [methylglyoxal] = 5.00 × 10^{-3} M, [2-mercaptoethanol] = 2.00 × 10^{-3} M. Buffer: (●) *N*-methylpyrrolidine at pH 10.40; (◐) (dimethylamino)ethanol at pH 9.05; (◑) *N*-ethylmorpholine at pH 7.71; (○) phosphate at pH 7.30.

Table IV. Third-Order Rate Constants for General-Base Catalysis

buffer	pK_a	reaction pH	slope (=αKk_{gb}), M^{-2} s^{-1}	k_{gb}, M^{-1} s^{-1}
HPO_4^{2-}	7.30	7.60	9.7 × 10^{-4}	4.85 × 10^{-3}
N-ethylmorpholine	7.71	7.71	1.50 × 10^{-3}	1.00 × 10^{-2}
(dimethylamino)-ethanol	9.40	9.05	1.05 × 10^{-2}	1.13 × 10^{-1}
N-methylpyrrolidine	10.40	10.40	3.95 × 10^{-1}	2.63
OH$^-$	15.7			285[a]
				328[b]
				295[c]
				343[d]

[a] From the slope of [HPO_4^{2-}] dependence. [b] From the slope of [*N*-ethylmorpholine] dependence. [c] From the slope of [(dimethylamino)ethanol] dependence. [d] From the slope of [*N*-methylpyrrolidine] dependence.

Examination of Table IV reveals that (i) the k_{OH} values determined at pH 7.6–10.4 are almost identical (315 ± 25 M^{-1} s^{-1}) and (ii) the k_{gb} value increases as increasing pK_a of buffer species. The Brønsted plots of pK_a vs. log k_{gb} provided a good linear relationship (r = 0.982, Figure 5), which is expressed by eq 6.

$$\log k_{gb} = 0.59\mathrm{p}K_a - 6.44 \qquad (6)$$

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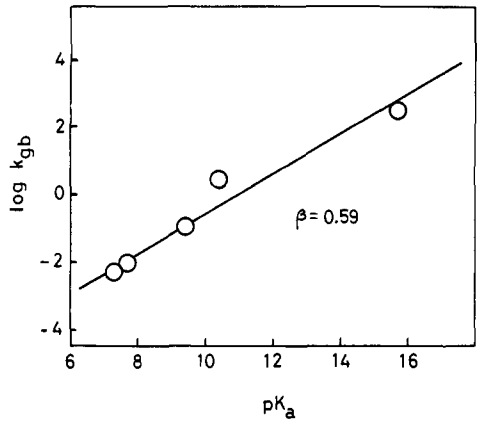

Figure 5. Brønsted plots.

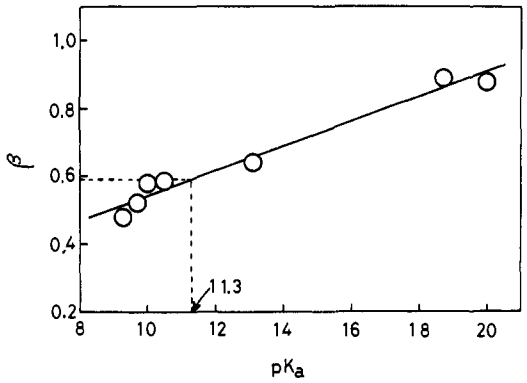

Figure 6. Plots of pK_a of carbon acids vs. β. The data are cited from ref 26.

In the general-base-catalyzed deprotonation of carbon acids, it has been reported that the magnitude of Brønsted slope (β) is associated with the acidity of the carbon acids.[26] We found that plots of pK_a of carbon acids against β provide a linear relationship given by eq 7 (see Figure 6), although the correlative coefficient

$$\beta = 0.036pK_a + 0.18 \quad (7)$$

is somewhat inferior ($r = 0.983$). On the basis of eq 7, one can roughly estimate the pK_a of **1** ($\beta = 0.59$) to be 11.3.

Flavin-Trapping Rate vs. Proton-Transfer Rate. The rearrangement of aliphatic glyoxals to **2** can be monitored by following the increase in the absorption band at 240 nm ($\epsilon = 3370$).[4] The method cannot be applied, however, to aromatic glyoxals and to borate buffer solutions, because aromatic glyoxals possess strong absorption bands at around 240 nm and borate buffer solutions do not give rise to the clear absorption increase at 240 nm. A question arises as to whether the flavin-trapping rate is equal to the proton-transfer rate in the absence of flavin. We thus determined by two methods the rate constants for the 2-mercaptoethanol plus methylglyoxal system under the identical reaction conditions. The kinetic measurement at 240 nm was also performed under anaerobic conditions, because 2-mercaptoethanol was oxidized by air under aerobic conditions, giving rise to a new absorption band at around 200 nm.

At pH 8.48, [2-mercaptoethanol] = 2.00×10^{-3} M, [methylglyoxal] = 5.00×10^{-3} M, and [MeFl] = 5.00×10^{-5} M; the rate (v_{obs}) calculated from zero-order decrease of the absorption band of MeFl was 2.50×10^{-9} M s^{-1}: that is, $k_{2,app} = k_2K = 2.50 \times 10^{-4}$ M^{-1} s^{-1} and $k_2 = 8.3 \times 10^{-4}$ sec^{-1}. On the other hand, the rate followed at 240 nm in the absence of MeFl satisfied the first-order kinetics (i.e., $v_{obsd} = k_2[\mathbf{1}]$), the k_2 being directly calculated from the slope of the first-order plot to be 8.43×10^{-4} s^{-1}. The results clearly support that the flavin-trapping rate is essentially equivalent to the proton-transfer rate.

Concluding Remarks. This study confirmed on the basis of a flavin-trapping technique that the rearrangement of hemithiol acetals to α-hydroxythiol esters occurs via 1,2-enediol intermediates as previously established by using NMR studies.[4] It is well-known that sulfur atom is able to stabilize the neighboring carbanion, facilitating the deprotonation from carbon acids.[27] It is concluded, therefore, that the acidity of hemithiol acetals is primarily due to the electron-withdrawing nature of acyl group and, in addition, is due to the stabilization of produced carbanions by thiol group. This would elucidate a role of GSH cofactor required by glyoxalase I.

Experimental Section

Materials. Thiophenol, 2-mercaptoethanol, and (dimethylamino)-ethanethiol were distilled under nitrogen stream before use. GSH was purchased from Wako Pure Chem. Ind. and assayed by the reaction with 4-nitrophenyl acetate.[28] 4-Chlorophenylglyoxal was prepared from 4-chloroacetophenone by SeO_2 oxidation;[29] mp 127–128 °C(lit.[29] 122 °C). Anal. ($C_8H_5Cl{\cdot}H_2O$) C, H, N. [1-^{2}H]Phenylglyoxal was obtained from [$^2H_3$]acetophenone.[4a,30] The ^{1}H NMR indicated that the content of acetophenone in the starting [$^2H_3$]acetophenone is less than 1%. *S*-Ethyl thiomandelate was prepared from phenylglyoxal and ethanethiol according to the Franzen's method;[5b] bp 115–119 °C (0.15mmHg) (lit.[5b] 125–126 °C (0.5mmHg)). The preparation of MeFl was described previously.[31]

Product Analyses. An aqueous solution (25% v/v of ethanol, pH 9.35 with 0.1 M borate buffer) containing thiol (65–130 mM), phenylglyoxal (13 mM), and MeFl (13 mM when required) was stirred in the dark under anaerobic (N_2) conditions, and the reaction was continued at 30 °C for 5 h. The solution was acidified with 1% HCl to pH 1–2 and was subjected to the high-pressure LC analysis (Shimadzu LC-3). When the elution was monitored at 254 nm, two peaks (except for that of MeFl) were detected for the sample solution obtained in the absence of MeFl. They were identified to be mandelic acid and benzoylformic acid. The sample solution obtained in the presence of MeFl gave a strong peak of benzolyformic acid, and a new small peak which was identified to be benzoic acid, but the peak of mandelic acid did not appear. The yields were determined by comparing the integrated intensity of the peaks with that of the authentic samples.

The decomposition of *S*-ethyl thiomandelate (17 mM) was conducted in an anaerobic aqueous solution (25% v/v of ethanol, pH 9.35 with 0.1 M borate buffer) containing MeFl (17 mM). After 5 h at 30 °C, the solution was acidified with 1% HCl to pH 1–2 and was subjected to high-pressure LC analysis. The integrated intensity of the peak of mandelic acid amounted to 84–95% of starting *S*-ethyl thiomandelate, and the peak of benzoylformic acid did not appear.

Kinetic Measurements. The kinetic measurements of the flavin oxidation were carried out at 30 °C in 25% v/v of aqueous ethanol under anaerobic conditions. A Thunberg cuvette was used to obtain the anaerobic reaction media. The details of the procedure were described previously.[9c] The typical example is as follows: a 3.0 mL aqueous buffer solution containing thiol was placed in a Thunberg cuvette, and 1.0 mL of ethanolic solution containing MeFl and glyoxal was deposited in a side arm of the cell. Both solutions were degassed for 20 min by bubbling N_2 through them, and, after being closed, the cuvette was equilibrated to 30 °C. The content of the side arm was rapidly mixed with the solution in the cell, and the progress of the reaction was monitored spectrophotometrically by following the disappearance of the absorption band of MeFl at 448 nm. In all the cases, the decrease of MeFl was zero order in MeFl. Introduction of oxygen into the final reaction mixture regenerated MeFl quantitatively.

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