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Equilibrium Constants for the Formation of Glyoxylate Thiohemiacetals and Kinetic Constants for Their Oxidation by O_2 Catalyzed by L-Hydroxy Acid Oxidase[1]

SANDRA GUNSHORE, EDWARD J. BRUSH, AND GORDON A. HAMILTON[2]

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

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Glyoxylate thiohemiacetal formation constants (defined as the concentration of thiohemiacetal divided by the concentration of thiol and the total concentration of hydrated and unhydrated glyoxylate) were determined at 25°C and pH 7.4 for a variety of thiols using two independent methods, and were found to be in the range of 0.2 to 1.7 mM^{-1}. Under the same conditions the hydration constant for glyoxylate (defined as the concentration of the hydrate divided by the concentration of the free aldehyde) was determined to be 163 ± 7. This information is used in conjunction with kinetic data to calculate kinetic constants for the oxidation of the thiohemiacetals by O_2 catalyzed by rat kidney L-hydroxy acid oxidase. The results further indicate that several such thiohemiacetals are excellent substrates, and suggest that one or more of them may be the physiological reactant for this enzyme.

INTRODUCTION

Recently we have reported (*1–8*) that adducts of various nucleophiles with glyoxylate are excellent substrates for the peroxisomal oxidases, D-amino acid oxidase, D-aspartate oxidase and L-hydroxy acid oxidase. Furthermore, we have summarized considerable circumstantial evidence indicating that such compounds are probably the physiological substrates for these mammalian enzymes. For L-hydroxy acid oxidase (*2–4*), the substrates are thiohemiacetals, the initial adducts formed on equilibration of thiols with glyoxylate (Eq. [1]). In the enzyme-catalyzed reaction they are converted to oxalyl thioesters (Eq. [2]).

$$RSH + HC(=O)-COO^- \rightleftharpoons RS-CH(OH)-COO^- \quad [1]$$

$$RS-CH(OH)-COO^- + O_2 \xrightarrow[\text{oxidase}]{\text{hydroxy acid}} RS-C(=O)-COO^- + H_2O_2 \quad [2]$$

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[2] To whom correspondence should be addressed.

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A prerequisite to the calculation of accurate kinetic constants for the hydroxy acid oxidase-catalyzed reaction is a knowledge of the equilibrium constants for thiohemiacetal formation. This is especially true if the thiol is an inhibitor or if it is otherwise impractical to use high concentrations of either the thiol or glyoxylate. Although equilibrium constants for the formation of thiohemiacetals from a number of other aldehydes have been reported (*9–14*), very little information is available with glyoxylate as the aldehyde. In this article we report the determination of such formation constants at 25°C and pH 7.4 for a variety of thiols using two independent methods. Both methods are based on measuring the rates of reactions that depend on the concentrations of one of the components of Eq. [1], thiol in one case and glyoxylate in the other. In either case the presence of the other component (glyoxylate or thiol) decreases the rate of the reaction due to the formation of thiohemiacetal, and the amount that it is decreased can be used to calculate the equilibrium constant for thiohemiacetal formation. One method is identical to that used by Kanchuger and Byers (*13*), and involves measuring the rate of the reaction of thiol with iodoacetamide (Eq. [3]). In the other method (Eq. [4]) the rate of reduction of glyoxylate by NADH catalyzed by lactate dehydrogenase (*15*) was followed under conditions where the rate is dependent on the glyoxylate concentration (nonsaturating conditions).

$$RSH + ICH_2CONH_2 \rightarrow RSCH_2CONH_2 + HI \qquad [3]$$

$$HC(=O)—COO^- + NADH + H^+ \xrightarrow[\text{dehydrogenase}]{\text{lactate}} HOCH_2COO^- + NAD^+ \qquad [4]$$

In addition to the formation constants for glyoxylate thiohemiacetals, the hydration constant for glyoxylate was also determined (by NMR techniques) and is reported. The various equilibrium constant data are then used, in conjunction with kinetic data for the enzyme-catalyzed reaction, to calculate apparent kinetic constants (using air saturated solutions of O_2) for the oxidation of several thiohemiacetals catalyzed by rat kidney L-hydroxy acid oxidase.

EXPERIMENTAL PROCEDURES

Materials

Unless otherwise noted, commercially available materials were used as received. The *o*-phthalic acid and picolinic acid, used as standards in the determination of the hydration constant for glyoxylate, were recrystallized twice (*o*-phthalic acid from water and picolinic acid from ethanol) and dried *in vacuo*. Crystalline rabbit muscle L-lactate dehydrogenase (935 units/mg) and crystalline beef liver catalase (30,000 units/mg) were obtained from Sigma Chemical Company. Rat kidney L-hydroxy acid oxidase was purified by the method of Cromartie and Walsh (*16, 17*); with DL-α-hydroxybutyrate as substrate it had initial activity comparable to that reported for the homogeneous enzyme.

Glyoxylate stock solutions were prepared from glyoxylic acid monohydrate, and their concentrations were determined by the bisulfite binding-iodometric titration method of Lewis and Weinhouse (*18*). DL-Dihydrolipoic acid was prepared by reduction of DL-lipoic acid with sodium borohydride (*19*), and D-pantetheine by the hydrogenation of D-pantethine (*20*). D-Pantetheine 4′-phosphate (phosphopantetheine) was generated (*21*) enzymatically from CoA by first hydrolyzing it to dephospho-CoA with potato acid phosphatase, and subsequently to phosphopantetheine and AMP using *Crotalus atrox* venom nucleotide pyrophosphatase. The preparation was shown by TLC to have only one thiol-containing compound that also had phosphate. Also, it was shown to be free of pantetheine, CoA, and dephospho-CoA. In all cases the concentrations of thiol solutions were determined by titration with 5,5′-dithiobis(2-nitrobenzoate) as described by Ellman (*22*), but assuming an extinction coefficient at 412 nm of 14.15 mM^{-1} (*23*) for the anion of thionitrobenzoate.

Methods

Hydration constant for glyoxylate. This constant (K_H), defined as in Eq. [5],

$$K_H = \frac{[(HO)_2CHCOO^-]}{[O{=}CHCOO^-]} \qquad [5]$$

was determined using a WM360 Bruker NMR spectrometer. Spectra (50 to 70 scans) were recorded using a 30-sec presaturation followed by a 3-sec acquisition. The temperature in the spectroscopic cell was 25.0 ± 0.1°C, and a coaxial tube filled with 99.8 atom% D_2O was used as an external lock. Because of the proximity of the large water peak to that of the α-H of the hydrate (at 4.9 ppm), it was not possible to measure the hydration constant by directly comparing the intensity of the free aldehyde proton (at 9.2 ppm) to that of the hydrate. Instead, spectra were obtained of solutions containing known amounts (0.1 to 0.6 M) of total glyoxylate (hydrated and unhydrated) and known amounts (3 to 5 mM) of an internal standard (*o*-phthalate or picolinate) in 0.1 M sodium phosphate buffer, pH 7.4. By comparing the integrated intensity of the glyoxylate aldehyde peak at 9.2 ppm to that of the *o*-phthalate peaks at 7.2 to 7.4 ppm (or the peaks at 8.4 or 7.4 to 7.6 ppm when picolinate is used as standard), the concentration of the free aldehyde form of glyoxylate can be determined. Thus, knowing the total concentration of glyoxylate, the hydration constant can be calculated.

Equilibrium constants for thiohemiacetal formation. All results were obtained at pH 7.4 using a 0.1 M sodium phosphate buffer solution containing 5 mM EDTA. Unless otherwise noted the temperature was 25.0°C. The equilibrium constant determinations that depend on the rate of reaction of free thiol with iodoacetamide were carried out as described by Kanchuger and Byers (*13*). Typically the reaction mixture consisted of thiol at 1.5 mM, iodoacetamide at 18 mM, and variable concentrations of total glyoxylate (0 to 38 mM). The progress of the alkylation reaction was monitored by diluting 50-μl aliquots of the reaction mixtures into cuvettes containing 0.60 mM 5,5′-dithiobis(2-nitrobenzoate) in a total volume of 2.0 ml.

For equilibrium constant determinations that involved the use of lactate dehydrogenase, solutions containing 2 mM total glyoxylate were incubated with 2 to 20 mM thiol in 1.0 ml total volume for 10 min. At the end of the incubation period, a 20-μl aliquot of an NADH solution was added to give a final concentration of 0.3 mM, the reaction was initiated by adding 20 μl of a lactate dehydrogenase solution (concentration in reaction mixture, 1.6 μg/ml), and the initial rate of NADH oxidation (to less than 5% reaction) was monitored at 340 nm. These rates were then used to calculate the concentration of glyoxylate available to the lactate dehydrogenase reaction by using a standard curve; the standard curve had been previously obtained by determining the rates for several different glyoxylate concentrations (from 0 to 2 mM) in the absence of added thiol but with other conditions remaining the same.

Knowing the total initial concentrations of thiol and glyoxylate and, after equilibration, the final free thiol or glyoxylate concentration, one has all the information needed to calculate the observed formation constant (K_F^{obs}), defined as in Eq. [6],

$$K_F^{obs} = \frac{[\text{thiohemiacetal}]}{[\text{RSH}]\,[\text{glyox}]_T} \quad [6]$$

where $[\text{glyox}]_T$ is equal to the sum of the concentrations of hydrated and unhydrated glyoxylate. The equilibrium constant (K_F) for the formation of thiohemiacetal from unhydrated glyoxylate is defined as in Eq. [7]. These equilibrium constants are related as shown in Eq. [8].

$$K_F = \frac{[\text{thiohemiacetal}]}{[\text{RSH}]\,[\text{O=CHCOO}^-]} \quad [7]$$

$$K_F = K_F^{obs}(K_H + 1) \quad [8]$$

L-*Hydroxy acid oxidase-catalyzed reactions*. The kinetic data were obtained by following oxygen consumption using a Clark oxygen electrode (Yellow Springs Instrument Co. Model 5331) connected to a monitor–amplifier and recorder. The reactions were run at 25°C with an air atmosphere ($[O_2]$ = 0.25 mM). The 3.0-ml reaction solution contained 0.1 M sodium phosphate, pH 7.5, 3 mM EDTA, 30 μg/ml catalase, and 63 μg/ml L-hydroxy acid oxidase. The enzymic reactions were usually initiated by adding an aliquot of the enzyme stock solution after the other components had temperature equilibrated 5 min. For the reactions involving the glyoxylate adducts of dihydrolipoate and propane-1,3-dithiol the reactions were initiated (*21*) by adding an aliquot of a glyoxylate stock solution after temperature equilibration. The rate of oxygen uptake was calculated from the initial rate (3–10% of completion) of disappearance of O_2. The observed rates of O_2 uptake were corrected for the nonenzymic autooxidation of the thiol, which is less than 5% of the enzymic rate, and were multiplied by a factor of two because catalase was present. The protein concentration was determined as previously described (*16*). In calculating k_{cat} it was assumed that there is one active site per 100,000 Da (*16*).

Different enzyme preparations had activities that varied from 3 to 7.4 mkat/kg (1 kat equals 1 mol/s) using 25 mM α-hydroxybutyrate as substrate. The values of k_{cat} given for the various substrates were normalized in order to account for the fact

that data with different substrates were obtained using enzyme preparations of differing specific activities. The standard was taken as 7.4 mkat/kg for the rate of oxidation with the substrate being 25 mM DL-α-hydroxybutyrate. Thus, if a particular enzyme preparation gave less than this rate with 25 mM hydroxybutyrate, the k_{cat}'s obtained for other substrates were increased by an appropriate factor so that the data with different substrates can be better compared.

RESULTS

Glyoxylate Hydration Constant

The hydration constant (K_H) for glyoxylate in water at 25°C and pH 7.4 was found by the NMR method described under Experimental Procedures to be 163 ± 7. This value is the average (± the standard deviation) of six separate experiments using two different internal standards (*o*-phthalate and picolinate). It differs considerably from values ranging from 15 to 32 that others had estimated (*14, 24–26*). However, all the previously employed methods for determining this constant are much less direct than the present NMR method and they involved the application of several assumptions. Consequently, the present value is considered to be more accurate.

Equilibrium Constants for Thiohemiacetal Formation

Summarized in Table 1 are formation constants at 25°C for glyoxylate thiohemiacetal formation determined using the lactate dehydrogenase method. One concern using this method is that some of the thiols or thiohemiacetals might inhibit the enzyme. The fact that the values calculated for the equilibrium constants are essentially the same when the thiol concentration is varied by fivefold or more argue against this possibility. However, to ensure that the lactate dehydrogenase method is giving reliable results, formation constants for a few of the thiols were determined using the less convenient thiol reactivity method, and these are summarized in Table 2. The generally good agreement between the results obtained by the two methods indicates that the values are reasonably accurate. Furthermore, the constants obtained (Tables 1 and 2) for the glutathione adduct are virtually identical to a value that can be calculated from published data (*14*).

A few of the formation constants were determined at 10° and 37°C, and these are summarized in Table 3. In general, thiohemiacetal formation is slightly less favored at higher temperatures. The data are not accurate enough to obtain good thermodynamic parameters for the equilibria, but the ΔH's are approximately -10 ± 2 kcal/mol and ΔS's approximately -20 ± 6 cal/mol-deg.

Kinetics of Glyoxylate Thiohemiacetal Oxidation Catalyzed by Hydroxy Acid Oxidase

In previous articles (*2–4*), we estimated approximate kinetic constants for the hydroxy acid oxidase-catalyzed oxidation of several glyoxylate thiohemiacetals

TABLE 1

EQUILIBRIUM CONSTANTS FOR GLYOXYLATE THIOHEMIACETAL FORMATION AS DETERMINED BY THE LACTATE DEHYDROGENASE METHOD[a]

Thiol	Thiol concentration range (mM)	K_F^{obs}[b] (mM^{-1})	K_F[c] (mM^{-1})
Glutathione	4–21	0.68 ± 0.05 (7)	112
Coenzyme A	2–10	0.77 ± 0.16 (6)	126
D-Pantetheine	3–13	1.11 ± 0.15 (11)	182
N-Acetylcysteamine	2–6	1.24 ± 0.12 (3)	203
2-Mercaptoethanol	4–10	0.68 ± 0.08 (6)	112
Ethanethiol	2–11	1.72 ± 0.14 (5)	282
2-Mercaptopropane	2–4	0.52 ± 0.04 (6)	85
N-(2-Mercaptopropionyl) glycine	3–7	0.57 ± 0.17 (3)	93
Mercaptosuccinate	7–29	0.21 ± 0.04 (6)	34
DL-Dihydrolipoate	3–12	0.97[d] ± 0.14 (13)	159[d]
Dithiothreitol	6–15	1.34[d] ± 0.14 (3)	220[d]
Dithioerythritol	6–15	1.58[d] ± 0.18 (3)	259[d]

[a] At 25.0°C in 0.1 M sodium phosphate buffer, pH 7.4, with 5 mM EDTA and 2 mM glyoxylate.

[b] Defined in Eq. [6]; each value with its standard deviation is the average of the number of experiments given in parentheses.

[c] Defined in Eq. [7].

[d] Because these compounds have two thiol groups, the equilibrium constants have been divided by a statistical factor of 2 so that the constants reflect the value per thiol group.

TABLE 2

EQUILIBRIUM CONSTANTS FOR GLYOXYLATE THIOHEMIACETAL FORMATION AS DETERMINED BY THE THIOL REACTIVITY METHOD[a]

Thiol	Glyoxylate concentration range (mM)	K_F^{obs}[b] (mM^{-1})	K_F[c] (mM^{-1})
Glutathione	18–38	0.71 ± 0.06 (3)	116
N-Acetylcysteamine	9–18	1.15 ± 0.04 (3)	189
2-Mercaptoethanol	18–38	0.90 ± 0.08 (13)	148
Mercaptosuccinate	18–38	0.12 ± 0.01 (6)	20

[a] At 25.0°C in 0.1 M sodium phosphate buffer, pH 7.4, with 5 mM EDTA and 1.5 mM thiol.

[b] Defined in Eq. [5]; each value with its standard deviation is the average of the number of experiments given in parentheses.

[c] Defined in Eq. [7].

TABLE 3

EFFECT OF TEMPERATURE ON THE EQUILIBRIUM CONSTANTS FOR GLYOXYLATE THIOHEMIACETAL FORMATION[a]

Thiol	Temperature (°C)	K_F^{obs} (mM^{-1})
Glutathione	10.0	2.04 ± 0.29 (3)
	25.0	0.68 ± 0.05 (7)
	37.0	0.33 ± 0.09 (4)
Coenzyme A	10.0	1.76 ± 0.24 (3)
	25.0	0.77 ± 0.16 (6)
	37.0	0.56 ± 0.05 (3)
2-Mercaptoethanol	10.0	3.07 ± 0.36 (3)
	25.0	0.68 ± 0.08 (6)
	37.0	0.58 ± 0.08 (7)

[a] Determined by the lactate dehydrogenase method under the same conditions as footnote *a*, Table 1, except that the temperature was varied as indicated.

by assuming that all the glyoxylate would be present as the thiohemiacetal when its total concentration is less than 1 mM and the thiol concentration is approximately 10 mM. With the present equilibrium constants one can now calculate the concentration of thiohemiacetal for any initial thiol and glyoxylate concentrations, and use this information to obtain Michaelis constants and catalytic rate constants from typical Lineweaver–Burk plots. Some results obtained in this way are summarized in Table 4. The constants are "apparent" constants obtained using an air atmosphere; the other substrate, oxygen, is not saturating under the reaction conditions (*2, 21*).

The constants listed in Table 4 for the glyoxylate adducts of phosphopantetheine and dihydrolipoate had to be determined by a slightly more complicated procedure because phosphopantetheine (*21*) and dihydrolipoate (*2*) themselves are competitive inhibitors of the hydroxy acid oxidase-catalyzed reaction. Thus, as one changes the glyoxylate or thiol concentration, the concentration of the inhibitor as well as of the thiohemiacetal substrate changes. One can see from the usual expression for competitive inhibition (Eq. [9]), however, that a straight line

$$\frac{1}{V} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}} \qquad [9]$$

should be obtained if $1/V$ is plotted versus $(1 + [I]/K_i)/[S]$. The inhibition constant K_i can be determined using hydroxybutyrate as substrate, and was found to be 0.13 mM for dihydrolipoate (*2*) and ca. 0.8 mM for phosphopantetheine (*21*). Since, using the present equilibrium constants, one can calculate the free thiol (I) and thiohemiacetal (S) concentrations for various ratios of thiol and glyoxylate, one thus has all the information to make the above plot. In Fig. 1 is illustrated such a plot for the data obtained using dihydrolipoate. The fact that a good straight line is obtained indicates that the data fit Eq. [9] quite well, and thus that the kinetic

TABLE 4

APPARENT KINETIC CONSTANTS USING AIR-SATURATED SOLUTIONS FOR SOME L-α-HYDROXY ACID OXIDASE-CATALYZED REACTIONS[a]

Substrate	k_{cat}[b] (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
DL-α-Hydroxybutyrate	0.76	0.60	1.27
Glyoxylate thiohemiacetal of:			
Coenzyme A	0.56	3.0	0.2
D-Phosphopantetheine	*ca* 0.9[c]	*ca* 2[c]	*ca* 0.4[c]
D-Pantetheine	0.67	0.67	1.00
N-Acetylcysteamine	1.42	0.42	3.4
2-Mercaptoethanol	1.03	0.59	1.74
DL-Dihydrolipoate	0.71[d]	0.007[d]	100[d]
Propane-1,3-dithiol	0.68[e]	0.021[e]	32[e]

[a] Data obtained at 25.0°C and pH 7.5 using air-saturated solutions. For most of the reactions involving thiol-glyoxylate adducts as substrates, the initial total glyoxylate concentration varied from 0.025 to 1.6 mM, the initial total thiol concentration from 1.0 to 30 mM, and thus the adduct concentrations varied from 0.023 to 1.4 mM. For the reaction involving dihydrolipoate and glyoxylate, the initial glyoxylate concentration ranged from 0.016 to 0.15 mM, the dihydrolipoate from 0.3 to 1.0 mM, and thus the adduct concentrations from 0.01 to 0.049 mM.

[b] Calculated assuming one active site per 100,000 Da (*16*), and normalized as described under Experimental Procedures.

[c] Calculated assuming the equilibrium constant for thiohemiacetal formation is the same as that for pantetheine.

[d] Calculated assuming the equilibrium constant for thiohemiacetal formation is $2 \times 0.97 = 1.94\ mM^{-1}$.

[e] Calculated assuming the substrate is the monothiohemiacetal and the equilibrium constant for its formation is twice that for ethanethiol, i.e., $2 \times 1.72 = 3.44\ mM^{-1}$.

constants obtained from the plot should be good estimates of the correct ones. Data obtained using phosphopantetheine also appear to fit such an equation but, because it was less extensively studied, the values for the kinetic constants listed in Table 4 should only be considered approximate (±25%).

DISCUSSION

As the equilibrium constant data for thiohemiacetal formation indicate (Tables 1 to 3), there is a strong tendency for thiols to add to glyoxylate. With a K_F^{obs} of approximately 1 mM^{-1}, this means, for example, that solutions containing 1 mM thiol and submillmolar concentrations of glyoxylate will have about half of the glyoxylate present as the thiohemiacetal. Consistent with the results of previous investigators using other aldehydes (*11–13*), the formation constant is largely independent of the acidity of the thiol, and it is not too dependent on structure

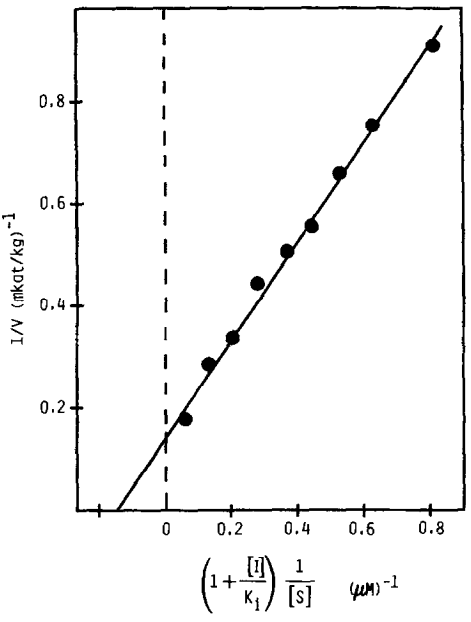


FIG. 1. A modified Lineweaver–Burk plot of data for the hydroxy acid oxidase-catalyzed reaction involving dihydrolipoate and glyoxylate. See the text and Table 4 for more details and a description of reaction conditions.

either, although the adducts from secondary thiols seem to be about threefold less stable than those from primary thiols (compare, for example, the constant for 2-mercaptopropane to that for ethanethiol). As a result the constant reported for dihydrolipoate is probably an average of a higher value for the 8-mercapto adduct and a lower value for the 6-mercapto adduct.

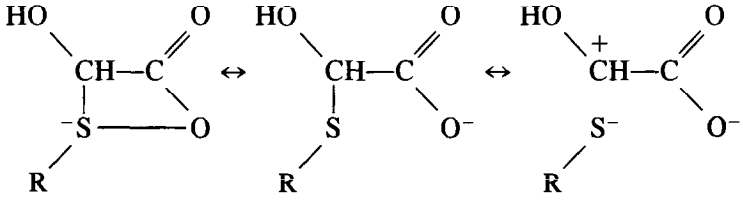
The stability of glyoxylate thiohemiacetals is considerably greater than one would have predicted from the known stability of thiohemiacetals formed from other aldehydes. Kanchuger and Byers (*13*) have shown that there is a linear correlation (slope close to one) between log K_F and log K_H for the addition of glutathione and water, respectively, to a series of aldehydes, but the point for the glutathione-glyoxylate adduct, calculated from the present data, falls considerably above this line. As the data in Tables 1 to 3 indicate, there is nothing unusual about the stability of the glyoxylate adduct formed from glutathione; those formed from other thiols have similar or greater stabilities. A measure of how much more stable the glyoxylate thiohemiacetal adducts are relative to those from other aldehydes can be gained by calculating the equilibrium constants for the reaction of Eq. [10]

$$\mathrm{GS{-}\underset{\displaystyle OH}{\underset{|}{CH}}{-}R + HO{-}\underset{\displaystyle OH}{\underset{|}{CH}}{-}COO^- \rightleftharpoons HO{-}\underset{\displaystyle OH}{\underset{|}{CH}}{-}R + GS{-}\underset{\displaystyle OH}{\underset{|}{CH}}{-}COO^-} \quad [10]$$

(GSH refers to glutathione). Some such equilibrium constants, defined as in Eq. [11] (subscript X refers to the constant for glyoxylate, and subscript R that for the

$$K_{eq} = \frac{[(HO)_2CHR][GSCH(OH)COO^-]}{[GSCH(OH)R][(HO)_2CHCOO^-]} = \frac{(K_F^{obs})_X[1 + 1/(K_H)_X]}{(K_F^{obs})_R[1 + 1/(K_H)_R]} \quad [11]$$

other aldehyde), are given in Table 5. The fact that K_{eq} is considerably greater than 1 suggests that the thiohemiacetal of glyoxylate has some special stability relative to other thiohemiacetals; the only other possibility is that the hydrate of glyoxylate is less stable than expected but that is not indicated by the observed K_H's. Disregarding the value for pyruvaldehyde (whose thiohemiacetal may also have extra stability), the average K_{eq} is 28 ± 10, which indicates a free energy difference of approximately 2 kcal/mol. It is not known what gives rise to this adduct stability, but it may be due to carboxylate charge delocalization into the sulfur as illustrated by structure **A**, or possibly that structure **B** is a major contributor to the resonance hybrid of the adduct. Delocalization as in **A** has been proposed to explain nucleophile–sulfur interactions in a number of crystal structures (*27*), and zwitterionic "Y" structures as encountered in **B** are also suspected to have special stability (*28*).



A **B**

Regardless of the reason for the stability of the glyoxylate thiohemiacetals, the fact that they are so stable means that reasonable quantities will be present in solution even when the thiol and glyoxylate are present at less than millimolar concentrations. This is one of the requirements that must be met in order for them to be possible physiological substrates for L-hydroxy acid oxidase. Another requirement is that they be efficiently converted by the enzyme, and the results summarized in Table 4 illustrate that several of them indeed are. One measure of how good a compound is as a substrate is k_{cat}, the maximum catalytic rate constant at saturating substrate concentrations, and it can be seen that all of the glyoxylate thiohemiacetals have apparent k_{cat}'s that are virtually identical to that for hydroxybutyrate, one of the best previously known substrates for the enzyme. However, k_{cat} does not account for the differing abilities of the substrates to bind to the enzyme. Consequently, perhaps the best measure of how good a compound is as a substrate at the relatively low concentrations expected *in vivo* is k_{cat}/K_m. By this measure several glyoxylate adducts of coenzyme A and its derivatives are comparable to hydroxybutyrate, and the dihydrolipoate-glyoxylate adduct is almost two orders of magnitude better. The constants reported for the dihydrolipoate-glyoxylate adduct are almost certainly composite constants, because glyoxylate could add to either the 8- or 6-mercapto group (presumably in approximately a 3 : 1 ratio), and it seems likely that each would be a substrate because the glyoxylate adducts of both 8- and 6-mercaptooctaonate are good

TABLE 5

EQUILIBRIUM CONSTANTS RELATING THE STABILITIES OF HYDRATES AND GLUTATHIONE THIOHEMIACETALS OF VARIOUS ALDEHYDES TO THOSE OF GLYOXYLATE

R of OCH—R	K_H[a]	K_F^{obs}[a] (mM^{-1})	K_{eq}[b]
$-COO^-$	163	0.69	—
$-CH_2CH_2CH_3$	0.48	0.010	23
$-CH_3$	1.26	0.012	32
$-CH_2OH$	7.9	0.036	17
$-CH_2NHCOCH_3$	9.0	0.013	48
$-CH_2OPO_3^{2-}$	11.7	0.038	17
$-CHOHCH_2OH$	21	0.018	37
$-CHOHCH_2OPO_3^{2-}$	27.5	0.033	20
$-CHOHCH_2OPO_3H^-$	300	0.025	28
$-CH_2N^+H_3$	840	0.023	30
$-COCH_3$	56 to 550	0.20	3.4

[a] Data for glyoxylate were taken from the present work and the other data from Ref. (*13*).

[b] Defined as in Eq. [11].

substrates (*7, 21*). In any event the oxalyl thioester products of the enzymic reaction involving dihydrolipoate would be expected to be in rapid equilibrium, as is the case for acetyl thioesters of dihydrolipoate (*29*).

The glyoxylate thiohemiacetal adducts are undoubtedly a mixture of isomers having the D and L configuration at the α-hydroxy position; a 50 : 50 mixture would be formed from nonchiral thiols, but it could be a different ratio for those adducts formed from chiral thiols. Since the D isomers of hydroxy acids are neither substrates nor inhibitors of L-hydroxy acid oxidase (*30, 31*), this means that the L isomers are even better substrates than the numbers in Table 4 imply. Another factor that will be involved in the binding of the substrates to the enzyme is how the equilibrium constant for thiohemiacetal formation is affected by removing the reactants from a water medium to a medium of lower dielectric constant, as expected on the surface of the enzyme. Recent evidence (*32*) indicates that this can have a several-fold adverse effect on the thiohemiacetal formation constant. Thus, it may be that the enzyme's inherent affinity for thiohemiacetals is much greater than the K_m values would suggest.

The observation that k_{cat}/K_m is so much higher for the dihydrolipoate-glyoxylate adduct than for the others might suggest that it is the most likely physiological reactant. However, other factors that must be taken into account are the expected physiological concentrations of the thiol and glyoxylate. The glyoxylate concentration in cells has been difficult to measure because of the reactions it undergoes, but best estimates suggest that it is probably on the order of 0.1 mM (*1, 7*). Thus, there is a sufficient amount present so that significant quantities of thiohemiacetals would be formed if the thiol is at approximately the same concentration. Coenzyme A and phosphopantetheine are present at greater than 0.1 mM concentra-

tions (*33–35*), so hydroxy acid oxidase-catalyzed reactions of their glyoxylate thiohemiacetals will almost certainly be occurring physiologically. On the other hand, the concentration of free dihydrolipoate in cells is probably on the order of 1 μM or less (*36*), so, despite the fact that its glyoxylate adduct is such a good substrate, it is unclear whether enough would be present for it to be a quantitatively significant substrate of the oxidase *in vivo*. It is possible, however, that the concentration of dihydrolipoate in peroxisomes, the organelle where the enzyme is located, may be higher, but that remains to be determined.

In summary then, the results of these and earlier (*2*) studies strongly imply that some thiol-glyoxylate adduct is the physiological substrate for rat kidney L-hydroxy acid oxidase. However, it may be too early to conclude definitely what specific thiol is the most important substrate *in vivo*. Of those studied, the most likely candidate is either dihydrolipoate or some coenzyme A derivative, but it is entirely possible that the reaction of some other physiological thiol not yet investigated may be more significant. Possible metabolic functions for the oxalyl thioester products of the enzymatic reaction have been discussed (*2, 7*).

Another conclusion from the present research is that, any time glyoxylate is in solution with thiols, a considerable amount will be present as the thiohemiacetal. This means that, in order to investigate the effects of glyoxylate on some particular enzyme system, it is important to control the level of thiols or otherwise the results will be meaningless. Also, since many enzymes have altered activities when reactive thiol groups on their surfaces are modified, one might expect that glyoxylate would be able to affect their activities by forming thiohemiacetals with such groups. In the accompanying paper (*37*) these effects are illustrated for one particular enzyme system.

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