Biol. 80, 41.
Stallcup, W. B., and Koshland, D. E., Jr. (1973b), *J. Mol. Biol. 80,* 63.
Stallcup, W. B., and Koshland, D. E., Jr. (1973c), *J. Mol. Biol. 80,* 77.
Stallcup, W. B., Mockrin, S. C., and Koshland, D. E., Jr. (1972), *J. Biol. Chem. 247,* 6277.
Stockell, A. (1959), *J. Biol. Chem. 243,* 1293.
Trentham, D. R. (1971), *Biochem. J. 122,* 59.
Velick, S. F. (1953), *J. Biol. Chem. 203,* 563.
Velick, S. F., and Hayes, J. E., Jr. (1953), *J. Biol. Chem. 203,* 545.
von Ellenrieder, G., Kirschner, K., and Schuster, I. (1972), *Eur. J. Biochem. 26,* 220.

Effects of pH and Thiols on the Kinetics of Yeast Glyoxalase I. An Evaluation of the Random Pathway Mechanism†

David L. Vander Jagt,*,‡ Elisabeth Daub, Jacqueline A. Krohn, and Liang-Po B. Han

ABSTRACT: The disproportionation of α-ketoaldehydes, catalyzed by yeast glyoxalase I, has been reported to involve a random pathway mechanism where one branch utilizes the hemimercaptal of glutathione and the α-ketoaldehyde in a one-substrate pathway, and the other branch utilizes first glutathione and then the α-ketoaldehyde in an ordered two-substrate pathway. The relative importance of the two pathways has been evaluated at 5° in the pH range 3–7, using methylglyoxal and phenylglyoxal as representive aliphatic and aromatic α-ketoaldehydes, by comparing initial rates of hemimercaptal formation in the absence of enzyme with initial rates of product formation in the presence of high enzyme concentrations. If the enzyme is not added last, the initial rates of product formation are the same as the initial rates of adduct formation even under conditions where it could be shown that dehydration of the hydrated α-ketoaldehyde is not entirely rate determining. If the enzyme is added after hemimercaptal formation, there is a "burst" of product formation equivalent to the amount of hemimercaptal, followed by a slower reaction, consistent with the one-substrate pathway. Additional support for this pathway was obtained from a study of the effects of added thiol reagents on the "burst" kinetics. The broad specificity of yeast glyoxalase I for both aliphatic and aromatic α-ketoaldehydes, reflected in V_{max} values which are insensitive to the nature of the α-ketoaldehyde, drops abruptly if the side chain of the α-ketoaldehyde is sterically crowded. The hemimercaptal of *tert*-butylglyoxal has a V_{max} 300-fold smaller than V_{max} for methylglyoxal; 2,4,6-trimethylphenylglyoxal is essentially inactive as a substrate even though the closely related compound 2,4-dimethylphenylglyoxal is a normal substrate. Analysis of the V_{max} and K_m (or K_i) values of these α-ketoaldehydes suggests that sterically crowded side chains affect both enzyme–substrate formation and the catalytic reaction.

The glyoxalase system catalyzes the disproportionation of α-ketoaldehydes, such as methylglyoxal, into α-hydroxycarboxylic acids. Two enzymic reactions take place: the first, catalyzed by glyoxalase I (*S*-lactoyl-glutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5), requires glutathione as cofactor and involves the conversion of an α-ketoaldehyde into a thiol ester of glutathione and the corresponding α-hydroxycarboxylic acid; the second reaction, catalyzed by glyoxalase II (*S*-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6), is the hydrolysis of the thiol ester to regenerate glutathione and liberate a free α-hydroxycarboxylic acid. Both a two-substrate mechanism (Kermack and Matheson, 1957) involving methylglyoxal (M) and glutathione (G) as substrates (I) and a one-substrate mechanism (Cliffe and Waley, 1961) involving the hemimercaptal (A) of methylglyoxal and glutathione as substrate (II) have been proposed for glyoxalase I. The one-substrate mechanism has received some additional experimental support (Davis and Williams, 1969).

$$v = \frac{V[M][G]}{K + K_m^G[M] + K_m^M[G] + [M][G]} \quad (1)$$

$$v = V[A]/(K_m^A + [A]) \quad (2)$$

Recently, Mannervik and coworkers carried out a detailed steady-state kinetic analysis of both yeast and porcine erythrocyte glyoxalase I using nonlinear regression methods to select the best of a number of mathematical models for glyoxalase I (Mannervik et al., 1973, 1974). Under most concentration conditions, the steady-state data can be fitted by either a one-substrate mechanism

$$v = \frac{V[A]}{K_m^A[1 + ([G]/K_i)] + [A]} \quad (3a)$$

with glutathione as a competitive inhibitor of the hemimercaptal or by a two-substrate ordered mechanism

$$v = \frac{V[M][G]}{K + K_m^M[G] + [M][G]} \quad (3b)$$

† From the Departments of Biochemistry and Chemistry, University of New Mexico, Albuquerque, New Mexico 87131. *Received March 14, 1975.* This work was supported by U.S. Public Health Service Grant CA 11850 from the National Cancer Institute and by a U.S. Public Health Service Research Career Development Award, CA 70939.

‡ Present address: Department of Biochemistry, University of New Mexico School of Medicine, Albuquerque, N.M. 87131.

Table I: Ultraviolet Data for the Hemimercaptals of α-Ketoaldehydes with Glutathione and for the Thiol Ester Products of the Glyoxalase I Reaction.[a]

α-Ketoaldehyde	$\lambda_{K_{diss}}$[b]	ϵ_{AD}[c]	ϵ_{KA}[c]	λ_R[d]	ϵ_R[e]	ϵ_P[e]
Methylglyoxal	240	440	<10	240	440	3300
Kethoxal	240	440	<10	240	440	4320
Hydroxypyruvaldehyde	240	360	<10	240	360	3950
tert-Butylglyoxal	240	750	<10	240	750	4070
Phenylglyoxal	280	2450	1280	263	6,790	1100
p-Methylphenylglyoxal	320	1250	165	273	10,300	1010
2,4-Dimethylphenylglyoxal	290	4420	2710	276	6,980	910
2,4,6-Trimethylphenylglyoxal	310	940	330			

[a] Data recorded at 25°, phosphate buffer, $\mu = 0.2$. [b] $\lambda_{K_{diss}}$ is the wavelength (nm) where the dissociation constants of the hemimercaptals were determined. [c] ϵ_{AD} and ϵ_{KA} are the molar extinction coefficients (M^{-1} cm^{-1}) for the hemimercaptals and the α-ketoaldehydes, respectively, at $\lambda_{K_{diss}}$. [d] λ_R is the wavelength where the glyoxalase I reaction was followed, which for the aromatic α-ketoaldehydes and their hemimercaptals is the isosbestic point. [e] ϵ_R and ϵ_P are the molar extinction coefficients of the hemimercaptals and the thiol ester products, respectively, at λ_R.

Table II: Initial Rates of Hemimercaptal Formation and Thiol Ester Product Formation for the Glyoxalase I Reaction with Methylglyoxal, 5°.[a]

pH[b]	[M] (m*M*)	[G] (m*M*)	V_{AD} (*M*/min)	V_P (*M*/min)
3.0	2.5	5.0	$0.82 \pm 0.12 \times 10^{-4}$	
	5.0	1.0	$0.44 \pm 0.06 \times 10^{-4}$	
	5.0	2.5	$0.92 \pm 0.14 \times 10^{-4}$	
	5.0	5.0	$1.6 \pm 0.2 \times 10^{-4}$	
5.0	1.0	5.0	$0.89 \pm 0.14 \times 10^{-4}$	
	2.5	2.5	$1.7 \pm 0.3 \times 10^{-4}$	
	5.0	0.5	$2.0 \pm 0.3 \times 10^{-4}$	$2.3 \pm 0.2 \times 10^{-4}$
	5.0	1.0	$2.6 \pm 0.4 \times 10^{-4}$	$3.0 \pm 0.3 \times 10^{-4}$
	5.0	2.5	$3.5 \pm 0.5 \times 10^{-4}$	$3.9 \pm 0.4 \times 10^{-4}$
	5.0	5.0	$3.7 \pm 0.6 \times 10^{-4}$	$4.0 \pm 0.4 \times 10^{-4}$
7.0	1.0	5.0	$1.7 \pm 0.3 \times 10^{-4}$	$2.0 \pm 0.2 \times 10^{-4}$
	2.5	2.5	$4.8 \pm 0.7 \times 10^{-4}$	$4.3 \pm 0.4 \times 10^{-4}$
	5.0	0.5	$9.2 \pm 1.4 \times 10^{-4}$	$9.1 \pm 0.9 \times 10^{-4}$
	5.0	1.0	$9.0 \pm 1.3 \times 10^{-4}$	$8.6 \pm 0.9 \times 10^{-4}$
	5.0	2.5	$9.5 \pm 1.4 \times 10^{-4}$	$9.3 \pm 0.9 \times 10^{-4}$
	5.0	5.0	$9.4 \pm 1.4 \times 10^{-4}$	$9.1 \pm 0.9 \times 10^{-4}$

[a] Methylglyoxal (M) and glutathione (G) react to form hemimercaptal with initial rates V_{AD} in absence of glyoxalase I. The reaction in the presence of high concentrations of glyoxalase I forms thiol ester product with initial rates V_P. [b] Formate, acetate, and phosphate buffers at pH 3.0, 5.0, and 7.0, respectively, all at $\mu = 0.2$.

requiring glutathione to add before methylglyoxal adds. Owing to the rapid equilibrium between methylglyoxal, glutathione, and hemimercaptal, eq 3a and 3b are kinetically equivalent

$$M + G \rightleftharpoons A$$

$$K_d = [M][G]/[A] \quad (4)$$

where $K_m^A = K/K_d$ and $K_i = K/K_m^M$. However, it was observed that at low concentrations of hemimercaptal and glutathione there was a hyperbolic curvature in Dixon plots of $1/v$ vs. [G] at various fixed levels of [A], leading to the proposal of an unusual random pathway mechanism (eq 5) which combines both the one- and two-substrate branches. The relative importance of the two branches could not be determined by the procedure used; nor could the question of whether eq 3a or 3b predominates at high reactant concentrations be answered by this type of kinetic analysis.

$$v = \frac{V_1[A] + V_2[A][M]}{K_1 + K_2[A][M] + K_3[G] + K_4[M] + [A]} \quad (5)$$

The present study is designed to evaluate the importance of the two branches of the random pathway mechanism for yeast glyoxalase I. In addition, results from studies on the range of α-ketoaldehydes which are substrates for glyoxalase I are reported. Yeast glyoxalase I has very broad specificity for both aliphatic and aromatic α-ketoaldehydes as reflected in V_{max} values which are quite insensitive to the nature of the α-ketoaldehyde (Vander Jagt et al., 1972a). This insensitivity of V_{max} is observed even though the intramolecular hydride migration step is rate-determining (Vander Jagt and Han, 1973). We report here some steric limitations to the broad specificity of glyoxalase I.

Experimental Section

Materials. Yeast glyoxalase I (Sigma) was obtained as a 50% glycerol solution and generally used directly. In other studies, further purification of the enzyme was carried out (Vander Jagt and Han, 1973). No indication of more than one form of the yeast enzyme has been observed. Glutathione (Sigma) was >99% pure by sulfhydryl titration with *N*-ethylmaleimide. Commercial methylglyoxal was purified by distillation. Kethoxal (β-ethoxy-α-ketobutyraldehyde) was a gift from the Upjohn Co, Kalamazoo, Mich. Hydroxypyruvaldehyde was prepared by the procedure of Reeves and Ajl (1965) involving cupric acetate oxidation of dihydroxyacetone. Phenylglyoxal and *p*-methylphenylglyoxal were prepared as previously described (Vander Jagt et al., 1972b). 2,4-Dimethylphenylglyoxal, 2,4,6-trimethylphenylglyoxal, and *tert*-butylglyoxal were prepared by oxidation of the corresponding methyl ketones with selenous acid (Riley and Gray, 1943).

Methods. Kinetics studies and determination of dissociation constants employed a Cary 15 recording spectrophotometer and a Gilford 222 modified Beckman DU, both temperature controlled with circulating water baths. pH measurements were made on a Sargent Welch Model DR pH meter with glass electrode. For first-order kinetics, rate constants were obtained from computer-calculated least-squares slopes of plots of log absorbance change vs. time. Correlation coefficients were generally better than 0.999.

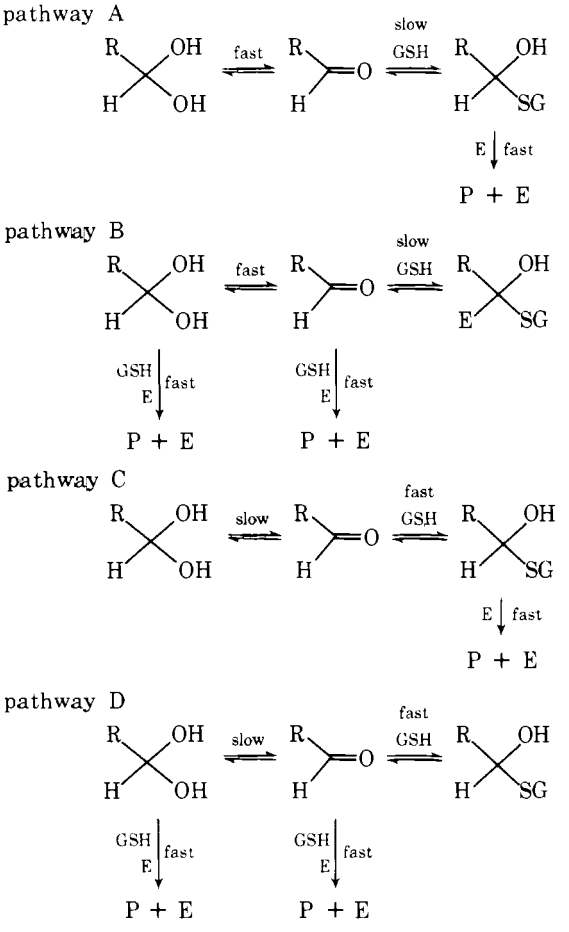
The dissociation constants of the hemimercaptals of glutathione and the α-ketoaldehydes are defined as: K_{diss} = [total α-ketoaldehyde][glutathione]/[hemimercaptal] where total α-ketoaldehyde is essentially equal to the concentration of the hydrated form, owing to the high degree of hydration of these very reactive aldehydes. The K_{diss} values were determined from spectral differences between the α-

ketoaldehyde hydrates and their hemimercaptals. Pertinent spectral data are listed in Table I. Initial rate studies in the glyoxalase I reaction were carried out as previously described (Vander Jagt et al., 1972a) by following thiol ester formation at 240 nm for the aliphatic α-ketoaldehydes and following loss of reactant at the apparent isosbestic point between α-ketoaldehyde and hemimercaptal for the aromatic α-ketoaldehydes (Table I). The kinetic constants for glyoxalase I reactions (Table III) were determined under conditions where the initial rates are proportional to the enzyme concentration. Initial rate studies at 5° (Table II) were measured as limiting rates in the presence of excess glyoxalase I.

Results

An evaluation of the random pathway mechanism of glyoxalase I for both the preenzymic and the enzymic reaction suggests there are four basic reaction pathways which need to be analyzed, as shown in Scheme I. Pathways A and

Scheme I



B represent one- and two-substrate pathways in which the preenzymic reaction involves rate-determining addition of glutathione to the free α-ketoaldehyde. Pathways C and D are the same as A and B except that the preenzymic reaction involves rate-determining dehydration of the hydrated α-ketoaldehyde. Both of the two-substrate pathways (B and D) are represented in Scheme I to indicate that glyoxalase I requires both glutathione and α-ketoaldehyde. However, there is the additional question of whether or not the unhydrated form of the α-ketoaldehyde is required.

In the first analysis of these pathways, initial rates of hemimercaptal formation (Table II), measured in the pH range 3–7 at 5° for the reaction of methylglyoxal with glutathione, were compared with the initial rates of thiol ester product formation measured with excess enzyme to give initial rates independent of enzyme concentration. At pH 7, within experimental error the initial rates of hemimercaptal formation and thiol ester formation are identical. In addition, the initial rates of hemimercaptal formation are independent of glutathione concentration, at pH 7. These data indicate that the preenzymic reaction involves rate-determining dehydration of methylglyoxal, thereby limiting the choice of mechanisms to pathways C and D (Scheme I). Furthermore, the identical initial rates of hemimercaptal and product formation suggest that if the two-substrate pathway (pathway D) is involved, the enzyme must utilize the unhydrated form of the α-ketoaldehyde.

Table III: Steric Effects in the Glyoxalase I Disproportionation of the Glutathione Hemimercaptals of a Series of Aliphatic and Aromatic α-Ketoaldehydes (pH 7.0).[a]

	K_M (M)	V_{MAX} (rel)[b]
Aliphatics		
Methylglyoxal	3×10^{-4}	1.00
Kethoxal	3×10^{-4}	0.75
Hydroxypyruvaldehyde	8×10^{-4}	0.43
tert-Butylglyoxal	2×10^{-4}	0.003
Aromatics		
Phenylglyoxal	2×10^{-4}	0.93
p-Methylphenylglyoxal	4×10^{-5}	0.27
2,4-Dimethylphenylglyoxal	4×10^{-5}	0.16
2,4,6-Trimethylphenylglyoxal		

[a] Phosphate buffer, 25°, $\mu = 0.2$. [b] All V_{MAX} values are relative to methylglyoxal.

Further studies on the limiting enzyme catalyzed reaction at pH 7 demonstrated that the order of addition of reactants has a dramatic effect on the initial rate. If the hemimercaptal reaction is allowed to proceed to equilibrium before glyoxalase I is added, the addition of enzyme produces an initial "burst" of product, followed by a slower reaction. Figures 1 and 2 are illustrative of the effects of order of addition of reactants; for either methylglyoxal or phenylglyoxal, as representative aliphatic and aromatic α-ketoaldehydes, the initial rates of hemimercaptal formation are identical with the initial rates of product formation if glyoxalase I is *not* added last, whereas reversal of the order of addition gives a "burst" of product. These results can be explained by the one-substrate pathway (pathway C). However, if both formation and breakdown of the hemimercaptals are rapid relative to dehydration of the α-ketoaldehydes, then the "burst" kinetics also are consistent with the two-substrate pathway (pathway D). The "burst" would simply indicate that unhydrated α-ketoaldehyde is produced more rapidly from hemimercaptal breakdown than from dehydration of hydrated α-ketoaldehyde. There are considerable data from the detailed studies of thiol additions to carbonyls by Jencks and coworkers to support the idea that both formation and breakdown of the hemimercaptals should be much faster than dehydration of the hydrated α-ketoaldehydes at pH 7 (Lienhard and Jencks, 1966; Barnett and Jencks, 1969). Consequently, the data in Figures 1 and 2 complement the pH 7 initial rate data in Table II, but these data do not allow one to evaluate the relative importance of the one- and two-substrate pathways.

The initial rates of hemimercaptal formation at acidic pH (Table II) are no longer independent of the glutathione

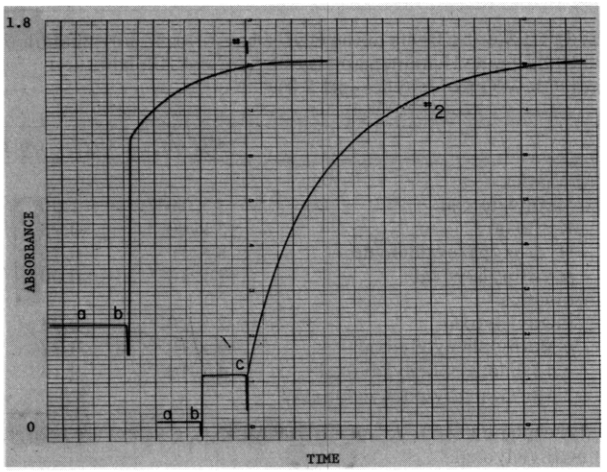


FIGURE 1: Disproportionation of methylglyoxal with high glyoxalase I concentrations. (1) The preenzymic reaction to form hemimercaptal (a) was carried out before enzyme was added (b). (2) First enzyme (a) and glutathione (b) were added before methylglyoxal was added (c). Reaction conditions were 5°, pH 7.0, 60 sec/div, 2.0 O.D. scale, 240 nm, [M] = 0.4 m*M*, [G] = 10 m*M*.

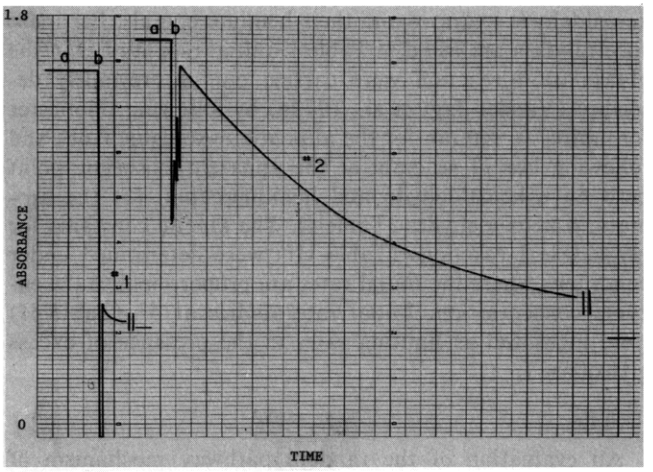
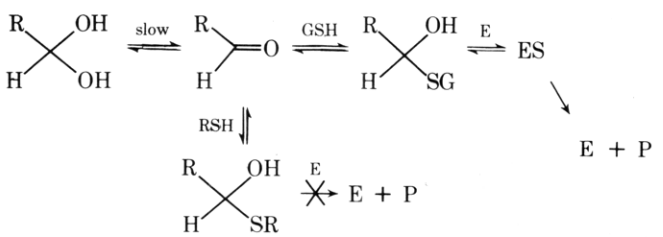


FIGURE 2: Disproportionation of phenylglyoxal with high glyoxalase I concentrations. (1) The preenzymic reaction to form hemimercaptal (a) was carried out before enzyme was added (b). (2) First enzyme and phenylglyoxal (a) were added before glutathione was added (b). Reaction conditions were 5°, pH 7.0, 20 sec/div, 2.0 O.D. scale, 263 nm, [PG] = 0.3 m*M*, [G] = 12 m*M*.

concentration. This is especially apparent at pH 3 where the initial rates are almost directly proportional to the glutathione concentration. At pH 5, the dependence of initial rates on glutathione concentration is less than at pH 3; a twofold change in initial rates is observed for a tenfold increase in glutathione. Although these results at pH 5 indicate that dehydration is still largely rate determining, the fact that a dependence on glutathione concentration is observed suggests that the dehydration of hydrated α-ketoaldehyde is somewhat faster than hemimercaptal formation. This provides a situation where the two-substrate pathway can be evaluated. One would expect that the initial rate of product formation would be faster than the initial rate of hemimercaptal formation for the two substrate mechanism. Within experimental error (Table II) the initial rates of hemimercaptal formation and thiol ester product formation are identical at pH 5, suggesting that the one-substrate pathway (pathway C) predominates under these experimental conditions. Admittedly, these results are not as definitive as one would like owing to the fact that dehydration is largely rate determining. The results would be more definitive at pH 3; however, yeast glyoxalase I is unstable below pH 5.

In order to obtain additional data for evaluating the relative importance of pathways C and D (Scheme I), a second experimental analysis was carried out at pH 7, 5°, by studying the effects of thiol reagents on the "burst" kinetics. The rationale for this approach is shown in Scheme II.

Scheme II[a]



[a] RSH = *N,N*-diethylcysteamine or *N*-acetylcysteine.

If the two-substrate mechanism predominates, the "burst" kinetics in Figures 1 and 2 would be interpreted to mean that hemimercaptal breakdown is much more rapid than dehydration. This also should apply to hemimercaptals formed from thiols other than glutathione. Thus one would expect "burst" kinetics if a non-glutathione hemimercaptal is present with glyoxalase I and the reaction is initiated by adding glutathione. There is also the possibility that at very high concentrations of thiol, the reaction to form non-glutathione hemimercaptal may compete with the enzyme reaction, leading to a less pronounced "burst".

In the one-substrate pathway, the presence of hemimercaptals formed from thiols other than glutathione should also function as sources of unhydrated α-ketoaldehyde which can react rapidly with glutathione, thereby providing "burst" kinetics. This "burst" should slow down markedly with increasing non-glutathione thiol concentration because glutathione would compete less effectively as the non-glutathione thiol concentration is raised. However, if glutathione is the only thiol present, one should observe "burst" kinetics which approach 100% of the reaction as the glutathione concentration is raised and there should be no slowdown in the "burst", if the one-substrate pathway predominates. Thus, this experimental analysis of pathways C and D is based upon the prediction that, in the "burst" kinetics, glutathione will behave differently than other thiols if the one-substrate pathway C predominates, but will behave similar to other thiols if the two-substrate pathway D predominates. Figures 3 and 4 are a comparison of the "burst" kinetics using glutathione and *N,N*-diethylcysteamine, respectively. Phenylglyoxal was used rather than methylglyoxal because the reaction could be followed at a higher wavelength where background absorption from the high thiol concentrations did not interfere. It is apparent that glutathione behaves differently, as predicted for a one-substrate mechanism. The results in Figure 4 for *N,N*-diethylcysteamine were also obtained if *N*-acetylcysteine was used. These two thiols have p*K* values which span the p*K* for the sulfhydryl group of glutathione, thereby ruling out the possibility that the results simply reflect differences in nucleophilicity. On the basis of the data in Figures 3 and 4, and the data in Table II, we conclude that the one-substrate pathway best describes the mechanism of yeast glyoxalase I under the conditions used in this study.

In these investigations, either methylglyoxal or phenyl-

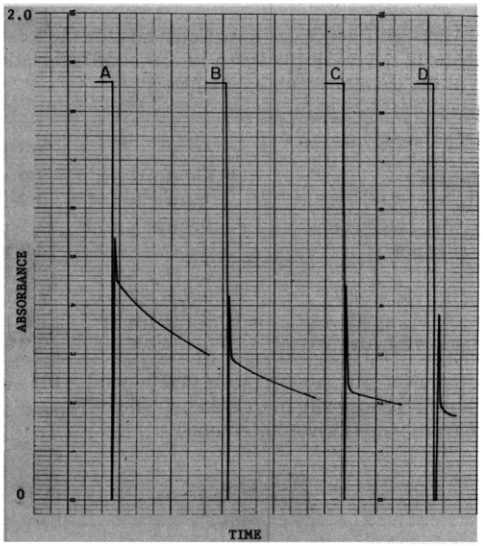


FIGURE 3: Disproportionation of phenylglyoxal with variable glutathione concentrations. In each reaction, glyoxalase-I was added last and [PG] = 0,3 m*M*. Glutathione concentrations were (A) 0.7 m*M*, (B) 1.7 m*M*, (C) 3.5 m*M*, (D) 40 m*M*. Reaction conditions were 5°, pH 7.0, 40 sec/div, 2.0 O.D. scale, 263 nm.

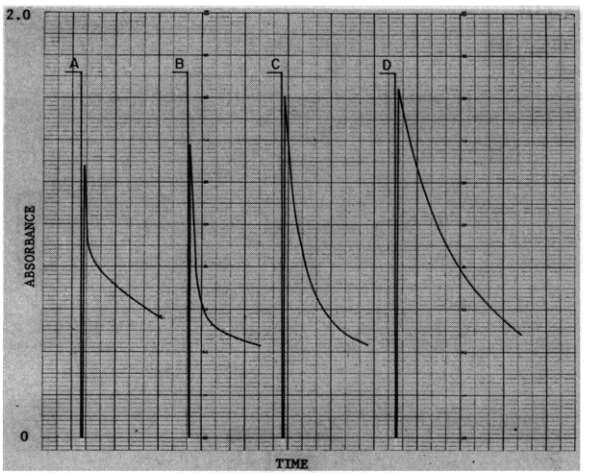
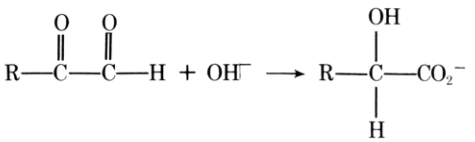


FIGURE 4: Disproportionation of phenylglyoxal with variable *N,N*-diethylcysteamine present. In each reaction, glyoxalase I and thiol were added to phenylglyoxal, and glutathione (0.7 m*M*) was added to initiate the reaction. *N,N*-Diethylcysteamine concentrations were (A) 0.8 m*M*, (B) 2 m*M*, (C) 8 m*M*, (D) 40 m*M*. Reaction conditions were 5°, pH 7.0, 40 sec/div, 2.0 O.D. scale, 263 nm.

glyoxal was used, depending upon experimental conditions, and it was assumed that the results obtained using either of these α-ketoaldehydes would be applicable to any conclusions made about the mechanism of yeast glyoxalase I. Previous studies (Vander Jagt et al., 1972a, 1973) on the kinetic parameters of yeast glyoxalase I indicated that these two substrates are very similar. To obtain additional data on these two α-ketoaldehydes, the stereospecificities of the enzyme reactions were studied. It is known that the glyoxalase system disproportionates methylglyoxal in a highly stereospecific manner to give D-lactic acid (Racker, 1951; Alexander, 1971). The glyoxalase I reaction was carried out using phenylglyoxal as a representative aromatic α-ketoaldehyde in order to compare the stereospecificity with that observed for methylglyoxal. Equivalent amounts of phenylglyoxal and glutathione were converted enzymically into the thiol ester of glutathione and mandelic acid. The thiol ester was hydrolyzed at pH 10 and acidified to pH 2, and the mandelic acid was extracted with ethyl ether. After removal of solvent, the residue was either sublimed or recrystallized from benzene. The optical rotation of the isolated material was compared with that of commercial D-mandelic acid at similar concentrations. The range of values obtained was:[1] isolated mandelic acid, $[\alpha]^{25}D$ -143 to $-151°$; commercial D-mandelic acid, $[\alpha]^{25}D$ -150 to $-155°$. The isolated mandelic acid had a melting point similar to D-mandelic acid and exhibited a slightly lower mixture melting point. Consequently, it appears that the glyoxalase I reaction is highly stereospecific (>95%) for aromatic as well as aliphatic α-ketoaldehydes. Glyoxalase I, by the one-substrate pathway, presumably works selectively on one of the two diastereomeric hemimercaptals to catalyze a stereospecific intramolecular hydride migration. This would not be inconsistent with identical initial rates of hemimercaptal and thiol ester formation in view of the rapid formation and breakdown of the hemimercaptals. The data in Figure 4 clearly support this conclusion, as do the studies of Jencks and coworkers.

Previous studies of the substrate specificity of yeast glyoxalase I indicated that a broad range of both aliphatic and aromatic α-ketoaldehydes can be disproportionated by the enzyme with little variation in V_{max} (Vander Jagt et al., 1972a). This contrasts with the glyoxalase model reaction of hydroxide ion with α-ketoaldehydes which is very sensitive to the variations in the side chain (Vander Jagt et al., 1972b):



(6)

The broad specificity of glyoxalase I falls off dramatically if the side chain of the α-ketoaldehyde is sterically crowded. Table III shows the K_M and V_{max} values for several aliphatic α-ketoaldehydes, all of which show similar chemistry in the preenzymic reaction as shown by the rates of hemimercaptal formation at pH 3.0 and the dissociation constants of the hemimercaptals at pH 7.0 (Table IV). The K_M and V_{max} values were obtained from double reciprocal plots assuming a one-substrate mechanism, described by eq 3a, and assuming that the substrate is the total hemimercaptal. The insensitivity of V_{max} is seen in methylglyoxal, kethoxal, and hydroxypyruvaldehyde where the range is only a factor of two. However, V_{max} for *tert*-butylglyoxal is some 300 times smaller than V_{max} for methylglyoxal, reflecting a marked steric effect. The K_M values for this series of hemimercaptals are quite similar. It was observed previously that the K_M values decrease with increasing apolar character of the side chain of the α-ketoaldehyde (Vander Jagt et al., 1972a). This is consistent with reports that the active site of yeast glyoxalase I appears to have an apolar binding site, based upon the observation that *S*-alkylglutathiones increasingly inhibit glyoxalase I as the length of the alkyl chain increases (Vince et al., 1971). The K_M value for hydroxypyruvaldehyde (8×10^{-4} *M*) is the largest K_M observed. This α-ketoaldehyde is also the most polar of any yet examined, and, consequently, its increased K_M is consistent with the suggestion of an apolar binding site.

[1] Commercial D-mandelic acid (Baker) is listed as $[\alpha]^{20}D$ -155 to $-150°$, similar to the experimental results in this study. Commercial L-mandelic acid (Baker) is listed as $[\alpha]^{20}D$ $+150$ to $+160°$.

Scheme III

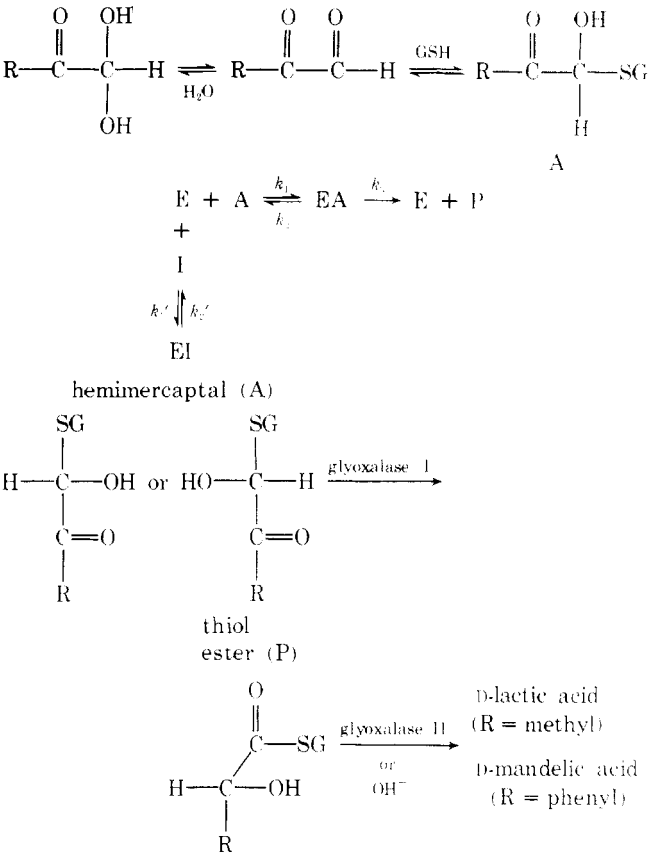


Table IV: Apparent First-Order Rate Constants for the Reaction of Glutathione with α-Ketoaldehydes (pH 3.0)[a] and Dissociation Constants of the Hemimercaptals (pH 7.0).[b]

α-Ketoaldehyde	k (sec^{-1})	K_{diss} (m*M*)[c]
Methylglyoxal	$6.6 \pm 0.4 \times 10^{-3}$	3.0 ± 0.5
Kethoxal	$4.3 \pm 0.1 \times 10^{-3}$	2.7 ± 0.1
Hydroxypyruvaldehyde	$5.4 \pm 0.4 \times 10^{-3}$	2.5 ± 0.7
tert-Butylglyoxal	$5.3 \pm 0.1 \times 10^{-3}$	2.3 ± 0.3
Phenylglyoxal	$7.2 \pm 0.1 \times 10^{-3}$	0.60 ± 0.05
p-Methylphenylglyoxal	$6.9 \pm 0.1 \times 10^{-3}$	1.1 ± 0.1
2,4-Dimethylphenylglyoxal	$10.3 \pm 0.4 \times 10^{-3}$	1.0 ± 0.1
2,4,6-Trimethylphenylglyoxal	$4.4 \pm 0.2 \times 10^{-3}$	0.94 ± 0.05

[a] Formate buffer, 25°, $\mu = 0.2$; [α-ketoaldehyde] = 0.3–0.4 m*M*; [GSH] = 5 m*M*. [b] Phosphate buffer, 25°, $\mu = 0.2$. [c] K_{diss} = [total α-ketoaldehyde] [GSH]/[hemimercaptal].

Table V: Disproportionation of Methylated Phenylglyoxals in Alkaline Solution.[a]

α-Ketoaldehyde	k (sec^{-1})
Phenylglyoxal	$7.60 \pm 0.13 \times 10^{-4}$
p-Methylphenylglyoxal	$3.05 \pm 0.05 \times 10^{-4}$
2,4-Dimethylphenylglyoxal	$91.2 \pm 2.9 \times 10^{-4}$
2,4,6-Trimethylphenylglyoxal	$813 \pm 13 \times 10^{-4}$

[a] Rates measured spectrophotometrically at the λ_{max} of the α-ketoaldehydes in phosphate buffer, pH 12, $\mu = 0.6$, 25°.

The K_M value for *tert*-butylglyoxal was examined further in order to determine if K_M for this α-ketoaldehyde is a true dissociation constant; $K_M = (k_2 + k_3)/k_1$ as defined in Scheme III. Since *tert*-butylglyoxal shows a V_{max} value much smaller than methylglyoxal, it was possible to use *tert*-butylglyoxal as a competitive inhibitor of methylglyoxal and obtain a dissociation constant, $K_i = k_2'/k_1'$ (Scheme III). Within experimental error, the value obtained for K_i is the same as K_M for *tert*-butylglyoxal. Therefore, $K_M = k_2/k_1 = K_S$ for this substrate, i.e., K_M is the dissociation constant of the enzyme–substrate complex. This is in contrast to methylglyoxal and phenylglyoxal where isotope effect studies have shown that $K_M = k_3/k_1$ (Vander Jagt and Han, 1973). Thus the K_M values for *tert*-butylglyoxal cannot be compared directly with the values for the other aliphatic substrates in Table III.

The glyoxalase I catalyzed disporportionation of the hemimercaptals of glutathione and aromatic α-ketoaldehydes also was examined for steric effects using a series of phenylglyoxals with increasing ring methylation. The preenzymic reaction again was normal (Table IV) for all of the compounds examined. The glyoxalase I reaction (Table III) shows broad specificity in V_{max} until two ortho substituents are present. 2,4,6-Trimethylphenylglyoxal is essentially inactive as a substrate for yeast glyoxalase I. 2,4-Dimethylphenylglyoxal is still a good substrate as reflected both in V_{max} and K_M. Thus this steric effect is quite subtle, appearing dramatically when a critical geometry of the side chain of the α-ketoaldehyde is encountered.

To establish whether the lack of reactivity of 2,4,6-trimethylphenylglyoxal as a substrate for glyoxalase I is the result of an inherent steric hindrance to the disporportionation reaction, the enzyme model reaction (eq 6) was studied for the aromatic α-ketoaldehydes. The results are in Table V. There is no inherent steric limitation but rather a potential steric acceleration for the disproportionation, evidenced by a greater than 100-fold increase in reactivity of 2,4,6-trimethylphenylglyoxal compared with phenylglyoxal.

Discussion

Comparisons of the initial rate data for hemimercaptal formation and product formation lead to several conclusions concerning the relative importance of the one- and two-substrate branches of the yeast glyoxalase I reaction. First, the data at pH 7 indicate that the two-substrate pathway requires the unhydrated α-ketoaldehyde. Second, the data at pH 5, where the dehydration step is somewhat faster than the rate of hemimercaptal formation, allow one to examine directly the relative importance of the two-substrate pathway. As at pH 7, the initial rates of hemimercaptal and product formation are identical, leading one to conclude that the two-substrate pathway is not the major contributor to the observed rates of reaction, under the experimental conditions used in this study. And third, the study of the "burst" kinetics at pH 7 in the presence of non-glutathione thiols allows one to show directly that the one-substrate pathway predominates. The random pathway mechanism was proposed as a result of measurements of steady-state rates at very low concentrations of hemimercaptal and glutathione where a hyperbolic curvature in Dixon plots was observed (Mannervik et al., 1973). At higher concentrations, the plots are linear and are described kinetically by either eq 3a or 3b. The results of the present study, based both upon failure to demonstrate a significant contribution from the two-substrate pathway and upon direct observation of the favorable substrate properties of the hemimercaptal using reversal of the order of addition of reactants,

demonstrate that eq 3a is an adequate expression of the kinetic data, at least at high substrate concentrations.

The steric effect observed in the disproportionation of α-ketoaldehydes with sterically crowded side chains suggests an interesting relationship between binding and catalysis, namely, that good substrates as measured by V_{max} also bind well, but poor substrates as measured by V_{max} bind poorly. The V_{max} for most α-ketoaldehydes studied to date fall in a 3–4-fold range (Vander Jagt et al. 1972a). These substrates show K_M values either comparable to that of methylglyoxal or somewhat smaller if the side chain is apolar. On this basis, one might expect *tert*-butylglyoxal and 2,4,6-trimethylphenylglyoxal to show K_M values lower than that of methylglyoxal and comparable to that of 2,4-dimethylphenylglyoxal. The K_M for *tert*-butylglyoxal is similar to that of methylglyoxal; however, $K_M = K_S$ for *tert*-butylglyoxal but not for methylglyoxal (Vander Jagt and Han, 1973). Since $K_M \geq K_S$, the K_M for *tert*-butylglyoxal, although not directly related to K_M for methylglyoxal, does allow one to conclude that the binding of *tert*-butylglyoxal is not as favorable as one might have expected for this apolar substrate. For 2,4,6-trimethylphenylglyoxal, this relationship between binding and catalysis is even more pronounced. This α-ketoaldehyde is disproportionated by glyoxalase I so slowly that meaningful kinetic parameters could not be obtained. It also binds very poorly based upon the observation that it is not a good competitive inhibitor of the other α-ketoaldehydes. This steric effect is not an inherent property of the substrate, based upon studies of the model reaction (eq 6).

References

Alexander, N. M., and Boyer, J. L. (1971), *Anal. Biochem. 41,* 29.

Barnett, R. E., and Jencks, W. P. (1969), *J. Am. Chem. Soc. 91,* 6758.

Cliffe, E. E., and Waley, S. G. (1961), *Biochem. J. 79,* 475.

Davis, K. A., and Williams, G. R. (1969), *Can. J. Biochem. 47,* 553.

Kermack, W. O., and Matheson, N. A. (1957), *Biochem. J. 65,* 48.

Lienhard, G. E., and Jencks, W. P. (1966), *J. Am. Chem. Soc. 88,* 3982.

Mannervik, B., Bartfai, T., and Gorna-Hall, B. (1974), *J. Biol. Chem. 249,* 901.

Mannervik, B., Gorna-Hall, B., and Bartfai, T. (1973), *Eur. J. Biochem. 37,* 270.

Racker, E. (1951), *J. Biol. Chem. 190,* 685.

Reeves, H. C., and Ajl, S. J/ (1965), *J. Biol. Chem. 240,* 569.

Riley, H. A., and Gray, A. R. (1943), Organic Synthesis, Collect. Vol. II, New York, N.Y., Wiley, p 509.

Vander Jagt, D. L., and Han, L.-P. B. (1973), *Biochemistry 12,* 5161.

Vander Jagt, D. L., Han, L.-P. B., and Lehman, C. H. (1972a), *Biochemistry 11,* 3735.

Vander Jagt, D. L., Han, L.-P. B., and Lehman, C. H. (1972b), *J. Org. Chem. 37,* 4100.

Vince, R., Daluge, S., and Wadd, W. B. (1971), *J. Med. Chem. 14,* 402.

Biodegradative Ornithine Decarboxylase of *Escherichia coli.* Purification, Properties, and Pyridoxal 5′-Phosphate Binding Site†

Deborah Applebaum,‡ Donna L. Sabo,‡ Edmond H. Fischer, and David R. Morris*

ABSTRACT: The biodegradative ornithine decarboxylase of *Escherichia coli* has been purified to apparent homogeneity. At its pH optimum (pH 7.0), the enzyme exists as a dimer of 160,000 molecular weight. Aggregation of the dimer was promoted by lower pH values. The enzyme requires pyridoxal 5′-phosphate for activity. The coenzyme appears to be bound in Schiff base linkage as suggested by spectral studies and inhibition by $NaBH_4$. The following sequence was determined for the coenzyme binding site: Val-His-(ε-Pxy)Lys-Gln-Gln-Ala-Gly-Gln. The properties of this enzyme are compared with the other biodegradative amino acid decarboxylases that have been isolated from *E. coli.*

Escherichia coli produces two distinct classes of L-amino acid decarboxylases which act, respectively, in biodegradative and biosynthetic roles (reviewed in Morris and Fillingame, 1974). The *biodegradative* decarboxylases are induced by growth at low pH in culture media enriched with amino acids (Gale, 1940). Production of the biodegradative decarboxylases seems to be a defense mechanism against low environmental pH. Bacterial mutants lacking the biodegradative arginine and histidine decarboxylases are unable to grow under acidic conditions (Becker, 1967; Recsei and Snell, 1972; Morris and Fillingame, 1974). The *biosynthetic* ornithine and arginine decarboxylases of *E. coli* are responsible for the synthesis of putrescine and, ultimately,

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received April 1, 1975.* This work was supported by grants from the National Institute of General Medical Sciences (GM-13957), the National Science Foundation (GB-3249), and the National Institute of Arthritis and Metabolic Diseases (AM-7902). It was taken in part from theses presented by D.A. and D.L.S. to the Graduate School of the University of Washington in partial fulfillment of the requirements for the Ph.D. degree.

‡ Supported by Training Grant GM00052 from the National Institute of General Medical Sciences.