Reisfeld, R. A., Lewis, V. J., and Williams, D. E. (1962), *Nature (London) 195*, 281.
Saari, J. C. (1972), Ph.D. Thesis, University of Washington, Seattle, Wash.
Saari, J. C., and Fischer, E. H. (1973), *Biochemistry 12*, 5225.
Sevilla, C. L., and Fischer, E. H. (1969), *Biochemistry 8*, 2161.
Shapiro, A. L., Viñuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Strausbauch, P. H., Kent, A. B., Hedrick, J. L., and Fischer, E. H. (1967), *Methods Enzymol. 11*, 671.
Ullmann, A., and Perrin, D. (1970), *in* The Lactose Operon, Beckwith, J. R., and Zipper, D., Ed., Cold Spring Harbor, N. Y., Cold Spring Harbor Laboratory, p 143.
Wang, J. H., and Graves, D. J. (1963), *J. Biol. Chem. 238*, 2386.
Wang, J. H., and Graves, D. J. (1964), *Biochemistry 3*, 1437.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Weiner, A. M., Platt, T., and Weber, K. (1972), *J. Biol. Chem. 247*, 3242.
Zarkadas, C. G., Smillie, L. B., and Madsen, N. B. (1968), *J. Mol. Biol. 38*, 245.
Zarkadas, C. G., Smillie, L. B., and Madsen, N. B. (1970), *Can. J. Biochem. 48*, 763.

Deuterium Isotope Effects and Chemically Modified Coenzymes as Mechanism Probes of Yeast Glyoxalase-I†

David L. Vander Jagt*,‡ and Liang-Po B. Han

ABSTRACT: The previously reported observation that the rates of disproportionation of the hemimercaptals of glutathione and substituted phenylglyoxals, catalyzed by yeast glyoxalase-I, are insensitive to substituents raised the question of whether or not the intramolecular hydride migration step is rate determining. This question has been investigated using deuterated α-ketoaldehydes. The disproportionation of methylglyoxal and perdeuteriomethylglyoxal shows an isotope effect on V_{max} ($V_{max,H}/V_{max,D} = 2.9$). This is comparable to the isotope effect observed in the hydroxide ion catalyzed disproportionation of methylglyoxal and perdeuteriomethylglyoxal, $k_H/k_D = 3.8$. Likewise, the glyoxalase-I-catalyzed disproportionation of phenylglyoxal and α-deuteriophenylglyoxal shows an isotope effect ($V_{max,H}/V_{max,D} = 3.2$) comparable to the hydroxide reaction ($k_H/k_D = 5.0$), leading to the conclusion that hydride migration is the rate-determining step for the glyoxalase-I reaction. For both pairs of α-ketoaldehydes, this isotope effect is also reflected in K_M, suggesting that the catalytic rate constant (k_3) is larger than the rate constant for dissociation of the enzyme–substrate complex (k_2) and that $K_M \simeq k_3/k_1$. Using purified preparations of glyoxalase-I, k_1 and k_3 were determined. The coenzyme role of glutathione in the glyoxalase-I reaction was evaluated using pH–rate profiles and chemically modified coenzymes. In the pH range 4.5–9, V_{max} shows no pH sensitivity; K_M values, however, increase at high and low pH suggesting that dissociable groups of pK values of about 5 and 8.5 are involved in binding the substrate to the enzyme. These apparent pK values are sensitive to the apolar character of the α-ketoaldehydes. V_{max} values are not affected if *N*-acetylglutathione is used in place of glutathione. K_M values, however, increase. Methylation of the glycyl residue of glutathione prevents binding of the hemimercaptals to the enzyme. Thus, the dissociable groups on glutathione appear to be involved primarily in enzyme–substrate formation rather than in the catalytic reaction.

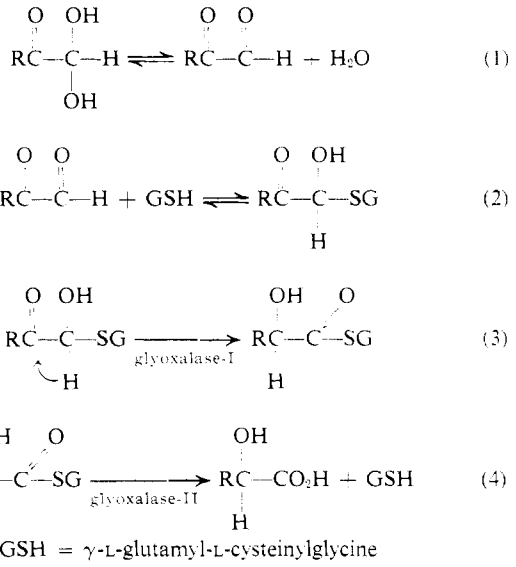
The glyoxalase system (Scheme I) which catalyzes the disproportionation of α-ketoaldehydes into the corresponding α-hydroxycarboxylic acids has been known for many years, although its biological role remains unclear. Several aspects of the mechanism of glyoxalase-I (*S*-lactoylglutathione methylglyoxal-lyase (isomerizing), EC4.4.1.5) which catalyzes the actual disproportionation reaction have been of interest, including the fact that the substrate for glyoxalase-I is a hemimercaptal formed in the preenzymic reaction between α-ketoaldehyde and coenzyme glutathione (Cliffe and Waley, 1961). Recent suggestions that the kinetic data may equally well be interpreted as indicating an ordered reaction requiring glutathione to bind before α-ketoaldehyde binds to the active site have been made (Bartfai *et al.*, 1973), but considerable other data are available which support Cliffe and Waley's original suggestion of Scheme I[1] (Davis and Williams, 1969; Vander Jagt *et al.*, 1972a). In our earlier studies on the substrate specificity of yeast glyoxalase-I, we reported that the reaction shows extremely broad specificity for the hemimercaptals of both aliphatic and aromatic α-ketoaldehydes and reported that V_{max} values are quite insensitive to variations in the α-ketoaldehyde (Vander Jagt *et al.*, 1972a). In particular, a series of substituted phenylglyoxals was examined for substituent effects on V_{max}. It was anticipated that the glyoxalase-I reaction, known to involve intramolecular hydride migration (Franzen, 1956; Rose, 1957), might be sensitive to the polarity of the α-ketone group and that the polarity of this group would be variable for a series of substituted phenylglyoxals. This was supported by studies of the disproportionation of

† From the Departments of Biochemistry and Chemistry, University of New Mexico, Albuquerque, New Mexico 87131. *Received August 22, 1973.* This work was supported by the U. S. Public Health Service under a grant from the National Cancer Institute (CA 11850). A preliminary report of this work was presented at the 164th National Meeting of the American Chemical Society, New York, N. Y., Aug 1972.

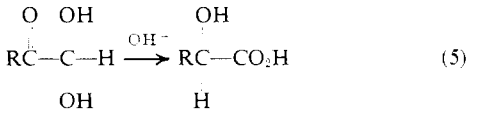
‡ Present address: Department of Biochemistry, University of New Mexico School of Medicine, Albuquerque, N. M. 87131.

[1] D. L. Vander Jagt, E. Daub, J. A. Krohn, and L.-P. B. Han, manuscript in preparation.

SCHEME I



substituted phenylglyoxals in alkaline solution (eq 5) which



involves rate-determining intramolecular hydride migration and is very sensitive to electron-withdrawing groups (Vander Jagt *et al.*, 1972b; Hine and Koser, 1971; Doering *et al.*, 1948). The absence of any significant substituent effect on V_{max} for the glyoxalase-I reaction raised the question of whether or not intramolecular hydride migration is rate determining in the enzyme reaction. We report here results from a study of some deuterium isotope effects to help determine the rate-determining step in the glyoxalase-I reaction and results from pH–rate studies and the use of chemically modified glutathiones to help determine the coenzyme role of glutathione in the reaction.

Experimental Section

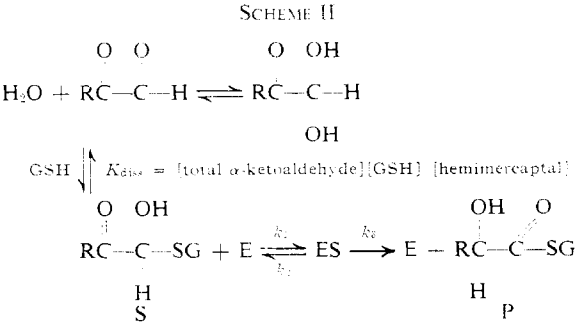
Materials. Yeast glyoxalase-I (Sigma) obtained as a 50% glycerol solution was purified on Sephadex G-100 to a specific activity of 1200 μmol/min per mg of protein, somewhat higher than the highest specific activity previously reported for the yeast enzyme (Davis and Williams, 1966). This material had a mol wt of about 35,000 determined from gel chromatography (lit. (Mannervik *et al.*, 1972) mol wt 33,000) and was homogeneous on disc gel electrophoresis. Glutathione (Sigma) was found >99% pure by sulfhydryl titration with *N*-ethylmaleimide (Alexander, 1958). Preparation of *N*-acetylglutathione by acetylation of glutathione in 90% formic acid with acetic anhydride (Utzinger *et al.*, 1963) afforded material with up to 90% theoretical sulfhydryl content as determined by titration with *N*-ethylmaleimide. Acetylation of the sodium salt of glutathione by adding acetic anhydride to an aqueous solution of the salt until no sulfhydryl groups remained, acidification with HCl, freeze drying, and crystallization of the residue from isopropyl alcohol afforded *N,S*-diacetylglutathione. Regeneration of the sulfhydryl group by brief treatment in alkaline solution conveniently gave solutions of *N*-acetylglutathione with at least 90% theoretical sulfhydryl content. Kinetic data obtained using either of these preparations were the same. Esterification of glutathione by passing HCl gas into a slurry of glutathione in methanol, 0°, until the reactant went into solution converted glutathione into a monomethyl ester; longer treatment introduced a second methyl group, as determined by nmr analysis. Chemical and spectral considerations suggest that the glycyl residue is esterified more rapidly than the γ-glutamyl residue. The amount of intact sulfhydryl was determined by titration with *N*-ethylmaleimide. Commercial methylglyoxal, 40% aqueous solution (Aldrich), was purified by distillation (Kermack and Matheson, 1957). Phenylglyoxal and *p*-chlorophenylglyoxal were prepared as previously reported (Vander Jagt *et al.*, 1972b). Perdeuteriomethylglyoxal was prepared by oxidation of perdeuterioacetone with selenous acid, followed by distillation. α-Deuteriophenylglyoxal was prepared by treating acetophenone with NaOD in a dioxane–D_2O solution to form acetophenone-d_3, followed by oxidation with selenous acid and distillation. The absence of any aldehydic proton was confirmed by nmr analysis.

Methods. DISSOCIATION CONSTANTS. The dissociation constants of the hemimercaptals formed in the preenzymic reaction were determined from spectral differences between the α-ketoaldehyde hydrates and the hemimercaptals, as discussed previously (Vander Jagt *et al.*, 1972a). Pertinent spectral data are listed in Table I.

KINETICS. Reaction rates for hemimercaptal formation at pH 3.0 and the rates of disproportionation of phenylglyoxal and α-deuteriophenylglyoxal in alkaline solution were monitored with a Gilford 222 modified Beckman DU recording spectrophotometer, temperature controlled with a circulating water bath. The rates of disproportionation of methylglyoxal and perdeuteriomethylglyoxal in alkaline solution were followed by taking samples, acidifying, and titrating the remaining α-ketoaldehyde with glyoxalase-I. Rate constants were obtained from computer-calculated least-squares lines of first-order plots; correlation coefficients were generally better than 0.999.

Analysis of the kinetics of the glyoxalase-I reaction from initial rate studies was carried out as before (Vander Jagt *et al.*, 1972a) by following thiol ester formation at 240 nm for the aliphatic α-ketoaldehydes and by following the loss of reactant at the apparent isosbestic point between α-ketoaldehyde and hemimercaptal for the aromatic α-ketoaldehydes. Pertinent spectral data are listed in Table I. The data were treated by the Michaelis–Menten scheme and V_{max} and K_M values obtained from double reciprocal plots (Lineweaver and Burk, 1934), assuming a one-substrate mechanism as in Scheme II, where

SCHEME II



$K_M = (k_2 + k_3)/k_1$ and $V_{max} = k_3[E]_0$.

The high pH studies of the enzyme reaction with methylglyoxal are complicated by the absorption contributed by the sulfide form of glutathione at 240 nm. However, by setting up

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

Glyoxal	Glutathione						*N*-Acetylglutathione					
	$\lambda_{K_{diss}}$[b]	ϵ_{Ad}[c]	ϵ_{KA}[c]	λ_R[d]	ϵ_R[e]	ϵ_p[e]	$\lambda_{K_{diss}}$	ϵ_{Ad}	ϵ_{KA}	λ_R	ϵ_R	ϵ_p
Methyl-	240	440	<10	240	440	3300	240	320	<10	240	320	2530
Perdeuteriomethyl-	240	430	<10	240	430	3580						
Phenyl-	280	2450	1280	263	6790	1100	280	3010	1280	263	5990	1060
α-Deuteriophenyl-	280	3020	1350	263	5690	950						
p-Chlorophenyl-	320	1050	140	270	10000	1020	320	1270	140	270	12,100	1,200

[a] Data recorded at 25°, phosphate buffer, pH 7.0, μ = 0.2, half of ionic strength from buffer and half from added KCl. [b] $\lambda_{K_{diss}}$ is the wavelength (nm) where the dissociation constants 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.

the preenzymic reaction using high [methylglyoxal]/glutathione], most of the glutathione is converted into the hemimercaptal, and the expression becomes $dP/dt = \Delta A/(\Delta t \cdot (\epsilon_p - \epsilon_{AD}))$ which is the same as the equation used at acidic and neutral pH values where the background absorbance of glutathione is small. For methylglyoxal, K_{diss} is pH insensitive in the range studied. Phenylglyoxal and *p*-chlorophenylglyoxal were monitored at their isosbestic points which are at high enough wavelength so that glutathione does not interfere. K_{diss} for the hemimercaptals of these α-ketoaldehydes increase somewhat at alkaline pH, going from 0.60 to 0.91 mM for phenylglyoxal and from 1.6 to 2.5 mM for *p*-chlorophenylglyoxal in the pH range studied.

Results

The hydroxide ion catalyzed disproportionation reaction (eq 5), which can be considered as a model reaction for the glyoxalase system, was examined for deuterium isotope effects. This reaction is known to involve rate-determining, intramolecular hydride migration (Vander Jagt *et al.*, 1972b; Hine and Koser, 1971; Doering *et al.*, 1948). At pH 12, the disproportionation of methylglyoxal and perdeuteriomethylglyoxal shows an isotope effect k_H/k_D = 3.8 (Table II). Owing to the possibility that the use of perdeuteriomethylglyoxal instead of α-deuteriomethylglyoxal may introduce secondary effects, phenylglyoxal was compared with α-deuteriophenylglyoxal where only a primary isotope effect exists. For phenylglyoxal and α-deuteriophenylglyoxal, k_H/k_D = 5.0 (Table II). These isotope effects give an indication of the magnitude one might expect for isotope effects in the glyoxalase-I reaction, if intramolecular hydride migration is also rate determining in the enzyme reaction (Scheme I).

The glyoxalase-I-catalyzed disproportionation of the hemimercaptals of methylglyoxal and perdeuteriomethylglyoxal shows an isotope effect on the maximal velocity ($V_{max,H}/V_{max,D}$ = 2.9) which is comparable to that observed in the model reaction. An isotope effect also is observed in the glyoxalase-I reaction with phenylglyoxal and α-deuteriophenylglyoxal ($V_{max,H}/V_{max,D}$ = 3.2). These data are summarized in Table III. On the basis of the observed isotope effect in the enzyme reaction and comparison of this isotope effect with that observed in the model reaction, one can conclude that intramolecular hydride migration is indeed the rate-determining step for glyoxalase-I, in spite of the absence of any significant sensitivity of V_{max} to variations in the side chain of the α-ketoaldehydes.

The deuterium isotope effect observed for V_{max} is also seen for K_M. For methylglyoxal and perdeuteriomethylglyoxal, $K_{M,H}/K_{M,D}$ = 1.7. This ratio is questionable because the effects of the several deuterium atoms on K_M might be expected to be more serious than their secondary isotope effect on V_{max}. This problem is not present in the comparison of phenylglyoxal and α-deuteriophenylglyoxal where $K_{M,H}/K_{M,D}$ = 3.3. This is, within experimental error, the same as the isotope effect on V_{max}. The data are summarized in Table III. Consideration of Scheme II suggests that if the isotope effect on V_{max} is the same as that on K_M, and if the isotope effect only indicates a change in the catalytic rate constant k_3, then $k_3 > k_2$ and $K_M \simeq k_3/k_1$. Thus, for the glyoxalase-I reaction, the Michaelis constant is considerably larger than the true dissociation constant for the enzyme–substrate complex ($K_S = k_2/k_1$). Using highly purified glyoxalase-I and assuming a mol wt of 35,000, the catalytic constant (turnover number) k_3 was determined from V_{max}: $k_3 = V_{max}/[E]_0 = 4.2 \times 10^4$ min^{-1}, where $[E]_0$ is the concentration of active site assuming one active site/mol wt 35,000. Using the K_M for the hemimercaptal of phenylglyoxal (2×10^{-4} M) and this value for k_3, one can calculate the rate constant, k_1, for formation of the enzyme–substrate complex, assuming $K_M = k_3/k_1$.

TABLE II: Deuterium Isotope Effects for the Base-Catalyzed Disproportionation of Methyglyoxal and Phenylglyoxal at pH 12.[a]

Glyoxal	$10^{-4}k$ (sec^{-1})	k_H/k_D
Methyl-	15.1 ± 0.2	3.8
Perdeuteriomethyl-	4.00 ± 0.02	
Phenyl-	7.60 ± 0.13	5.0
α-Deuteriophenyl-	1.52 ± 0.02	

[a] Phosphate buffer, μ = 0.6, 25°. Methylglyoxal and perdeuteriomethylglyoxal were determined by taking samples and titrating the remaining α-ketoaldehyde at pH 7 using the glyoxalase system; phenylglyoxal and α-deuteriophenylglyoxal were determined spectrophotometrically at 251 and 252 nm, respectively.

TABLE III: Deuterium Isotope Effects on the Kinetic Parameters K_M and V_{max} for the Glyoxalase-I-Catalyzed Disproportionation of Methylglyoxal and Phenylglyoxal.

Glyoxal	$10^4 K_M$ (M)	$K_{M,H}/K_{M,D}$	V_{max}[a]	$V_{max,H}/V_{max,D}$
Methyl-	3.2 ± 0.2	1.7 ± 0.2	1.00 ± 0.05	2.9 ± 0.2
Perdeuteriomethyl-	1.9 ± 0.2		0.34 ± 0.05	
Phenyl-	2.0 ± 0.2	3.3 ± 0.5	0.94 ± 0.05	3.2 ± 0.3
α-Deuteriophenyl-	0.60 ± 0.04		0.29 ± 0.05	

[a] V_{max} values all relative to methylglyoxal; all data at pH 7.0, 25°, phosphate buffer, μ = 0.2, half of ionic strength from buffer and half from added KCl.

$$k_1 = \frac{k_3}{K_M} = \frac{4.2 \times 10^4 \text{ min}^{-1}}{2 \times 10^{-4} \text{ M}} = 2.1 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$$

This value, 2.1×10^8 M^{-1} min^{-1} or 3.5×10^6 M^{-1} sec^{-1}, is considerably less than a diffusion-controlled rate constant for the binding of molecules of the size of these hemimercaptals to the active site of glyoxalase-I. However, it is similar to a number of binding constants which have been reported for a variety of systems using pre-steady-state measurements (Hammes and Schimmel, 1970). If the conclusion that $K_M = k_3/k_1$ is fairly general for all substrates for glyoxalase-I, then, in view of the fact that k_3 shows little sensitivity to variation in substrate, most of the observed specificity of the glyoxalase-I reaction must reside in k_1, that is, in the rate of enzyme–substrate complex formation. It was observed, previously, that increasing apolar character of the α-ketoaldehydes resulted in smaller K_M values (Vander Jagt *et al.*, 1972a). The present results suggest that this is the result of increasing values of k_1 as the apolar character of the substrate increases.

The effects of pH on the kinetic parameters K_M and V_{max} were determined for methylglyoxal, phenylglyoxal, and *p*-chlorophenylglyoxal. For all three of these α-ketoaldehydes, V_{max} is insensitive to pH (Figures 1, 2, and 3), while K_M values increase both at high and at low pH. The lower end of the pH range examined was dictated by the stability of the enzyme. The upper pH was limited by the experimental procedure; for methylglyoxal, the background absorbance at 240 nm contributed by glutathione interferes at high pH even though the preenzymic equilibrium was set up to minimize the amount of free gultathione. For phenylglyoxal and *p*-chlorophenylglyoxal, K_{diss} increases rapidly near pH 9 and precludes setting up the preenzymic reaction at the isosbestic wavelength with sufficient concentrations of the hemimercaptals to allow initial rate measurements at substrate concentrations which approach K_M. The lack of a pH effect on V_{max} indicates that, in the pH range examined, no dissociable groups are encountered which are important in the conversion of the enzyme–substrate complex into product. However, the pH influence on K_M suggests that, at both high and low pH, dissociable groups are encountered which are important for binding the substrate to the active site. Using the procedure of Dixon and Webb of plotting log V_{max}/K_M *vs.* pH, the intersection of lines of integral slope should indicate the apparent pK values of groups involved in substrate binding (Dixon and Webb, 1964). Figures 1, 2, and 3 indicate the participation of groups with pK values of 4.7–5.2 and 8.4–8.9 depending upon the apolar character of the α-ketoaldehydes. Thus, the pK

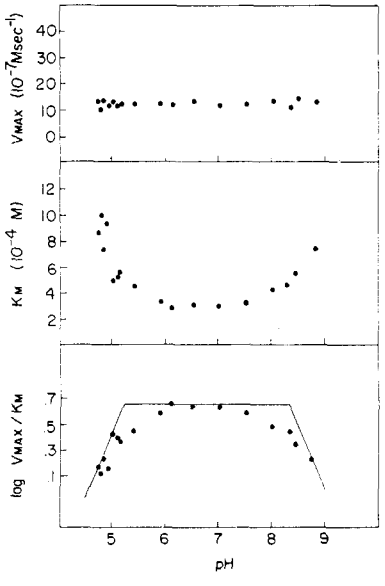


FIGURE 1: pH profiles for the glyoxalase-I-catalyzed disproportionation of the glutathione hemimercaptal of methylglyoxal, 25°. The pH range used acetate, phosphate, and carbonate buffers, μ = 0.2, half from buffer and half from added KCl.

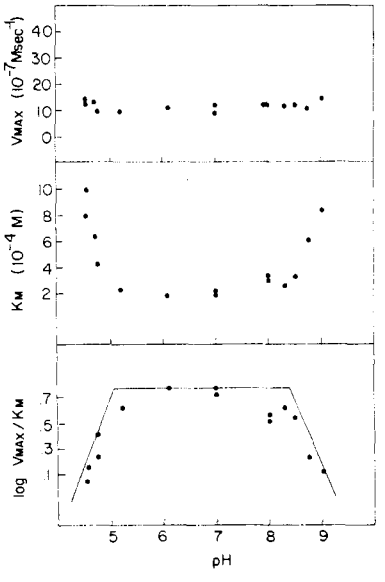


FIGURE 2: pH profiles for the glyoxalase-I-catalyzed disproportionation of the glutathione hemimercaptal of phenylglyoxal, 25°.

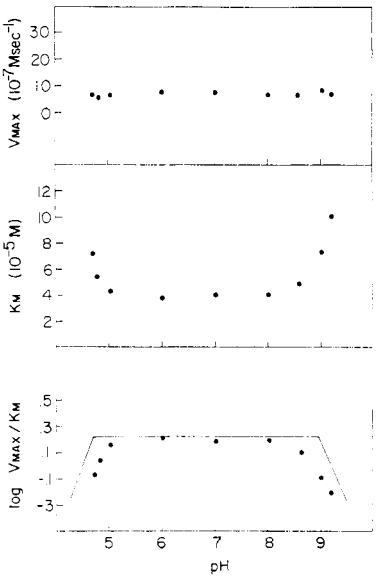


FIGURE 3: pH profiles for the glyoxalase-I-catalyzed disproportionation of the glutathione hemimercaptal of *p*-chlorophenylglyoxal, 25°.

values observed when *p*-chlorophenylglyoxal is the α-ketoaldehyde of choice are lower on the acid side and higher on the alkaline side (Figure 3) compared to the values for methylglyoxal (Figure 1); phenylglyoxal is intermediate (Figure 2).

The pK values determined from log V_{max}/K_M plots are tentatively assigned to dissociable groups on the enzyme, rather than to groups on glutathione. Considerable data are available on the ionization scheme of glutathione and related compounds (Vander Jagt *et al.*, 1972c; Martin and Edsall, 1958). The pK values of the two carboxylic acid groups of glutathione are 2.12 and 3.73, both considerably more acidic than the pK 4.7–5.2 obtained from the log V_{max}/K_M plots. The pK values for the SH and NH_3^+ proton dissociations of glutathione cannot be assigned directly from the apparent pK values obtained from potentiometric titration of glutathione, owing to the fact that these two dissociations overlap and consequently give pK values which are composites of the two ionizations. However, the microionization constants have been determined for these groups. Those which apply to ionization of the α-amino group of the glutamyl residue are in the pH range 9.4–10. Admittedly, the possibility should be considered that the pK values of glutathione are markedly altered when glutathione exists as a hemimercaptal. This seems unlikely based upon available data for glutathione. Hence, the pK 8.4–8.9 from the log V_{max}/K_M plots would appear to be too low to be assigned to glutathione, although this is less certain than the conclusion that the pK value 4.7–5.2 is not from glutathione.

The role of glutathione in the glyoxalase-I reaction was examined by using chemically modified glutathiones. *N*-Acetylglutathione (*N*-acetyl-GSH) and glutathione esterified by methylation of the glycyl residue (GSH-OCH_3) were compared with glutathione (GSH), both in the preenzymatic reaction and in the enzyme reaction. In the preenzymic reaction, the modified glutathiones do not significantly affect the rate of hemimercaptal formation at pH 3.0 (Table IV). Hemimercaptal formation requires loss of water (Scheme I) before the thiol can add to the aldehyde carbonyl in a reaction which goes to equilibrium. However, the conditions used were designed to push the reaction toward completion; the rate under these conditions is insensitive to different α-ketoaldehydes or modified glutathiones (Table IV).

TABLE IV: Apparent First-Order Rate Constants for the Reactions of Glutathione, *N*-Acetylglutathione, and Methylglutathione with α-Ketoaldehydes (pH 3.0).[a]

Glyoxal	Thiol	10^3k (sec^{-1})
Methyl-	GSH	6.5 ± 0.4
	N-Acetyl-GSH	7.7 ± 0.7
	GSH-OCH_3	11 ± 1
Perdeuteriomethyl-	GSH	6.3 ± 0.3
	N-Acetyl-GSH	11 ± 1
Phenyl-	GSH	7.2 ± 0.1
	N-Acetyl-GSH	6.8 ± 0.7
	GSH-OCH_3	9.3 ± 0.9
α-Deuteriophenyl-	GSH	8.3 ± 0.2
	N-Acetyl-GSH	6.6 ± 0.7
p-Chlorophenyl-	GSH	7.6 ± 0.2
	N-Acetyl-GSH	5.9 ± 0.6

[a] Formate buffer, 25°, μ = 0.2, half of ionic strength from buffer and half from added KCl; [α-ketoaldehyde] = 0.3–0.4 mM; [thiol] = 5 mM.

The GSH-OCH_3 derivative is inactive in the glyoxalase-I reaction even though the preenzymic reaction is normal. No disproportionation is observed for the hemimercaptals of various α-ketoaldehydes and GSH-OCH_3. Furthermore, hemimercaptals of GSH-OCH_3 do not inhibit the glyoxalase-I disproportionation of hemimercaptals of glutathione, suggesting that esterification of the glycyl residue markedly affects the formation of the enzyme–substrate complex.

The *N*-acetyl-GSH derivative is active in the glyoxalase-I reaction. Using the K_{diss} values for the preenzymic reaction listed in Table V, the enzyme reaction was examined for methylglyoxal, phenylglyoxal, and *p*-chlorophenylglyoxal. Kinetic parameters are listed in Table VI. The V_{max} values with glutathione as coenzyme compared with *N*-acetyl-GSH are within experimental error identical, indicating that the α-amino group of the glutamyl residue of glutathione is not involved in the catalytic step, k_3 (Scheme II). However, for all

TABLE V: Dissociation Constants, K_{diss}, of the Hemimercaptals Formed in the Preenzymic Reaction between α-Ketoaldehyde and Coenzyme.[a]

Glyoxal	Coenzyme	K_{diss} (mM)[b]
Methyl-	GSH	3.0 ± 0.5
	N-Acetyl-GSH	1.0 ± 0.1
Perdeuteriomethyl-	GSH	3.4 ± 0.2
Phenyl-	GSH	0.60 ± 0.05
	N-Acetyl-GSH	0.9 ± 0.2
α-Deuteriophenyl-	GSH	1.6 ± 0.2
p-Chlorophenyl-	GSH	1.6 ± 0.5
	N-Acetyl-GSH	1.6 ± 0.5

[a] Phosphate buffer, 25°, μ = 0.2, half of ionic strength from buffer and half from added KCl. [b] K_{diss} is defined in the Experimental Section.

TABLE VI: Kinetic Parameters K_M and V_{max} for the Glyoxalase-I Reaction with Glutathione and *N*-Acetylglutathione as Coenzymes.

Glyoxal	Coenzyme	$10^4 K_M$ (M)[a]	V_{max} (rel)
Methyl-	GSH	3.2 ± 0.2	1.0 ± 0.05[b]
	N-Acetyl-GSH	40 ± 5	1.0 ± 0.1
Phenyl-	GSH	2.0 ± 0.2	0.9 ± 0.05
	N-Acetyl-GSH	10 ± 3	0.8 ± 0.2
p-Chlorophenyl-	GSH	0.42 ± 0.02	0.5 ± 0.05
	N-Acetyl-GSH	2 ± 0.5	0.4 ± 0.1

[a] K_M values for the hemimercaptals formed in the preenzymic reaction between α-ketoaldehyde and coenzyme. [b] V_{max} values all relative to GSH with methylglyoxal; all data at pH 7.0, 25°, phosphate buffer, μ = 0.2, half of ionic strength from buffer and half from added KCl.

three α-ketoaldehydes examined, K_M values increase when *N*-acetyl-GSH replaces glutathione, suggesting that the amino group may be important in enzyme–substrate formation. The high K_M values with *N*-acetyl-GSH make kinetic analysis difficult because the experimental conditions limited the concentrations of hemimercaptal one could set up in the preenzymic reaction. The data in Table VI were determined at a concentration range of hemimercaptal which was below K_M. This introduces some uncertainty in the parameters K_M and V_{max}. The values for V_{max} listed in Table VI for comparisons of glutathione and *N*-acetylglutathione are within experimental error the same. This experimental limitation precluded carrying out pH–rate studies with *N*-acetyl-GSH as coenzyme.

Discussion

The glyoxalase-I reaction proceeds by a rate-determining intramolecular hydride migration step, in light of the isotope effects observed in the present study. The absence of any significant sensitivity of V_{max} to variations in the side chain of the α-ketoaldehydes raises the question of why this intramolecular step is insensitive to the polarity of the α-ketone group. The model reaction of substituted phenylglyoxals in alkaline solution is very sensitive to the polarity of the α-ketone group (Vander Jagt *et al.*, 1972b). Davis and Williams (1966) were the first to observe that yeast glyoxalase-I is a metalloenzyme (Mg^{2+}) and to suggest that the metal ion may be involved at the active site to complex the ketone carbonyl oxygen and the hemimercaptal oxygen in a reaction similar to an intramolecular Meerwein–Pondorf–Oppenauer reaction (Woodward *et al.*, 1945). Interestingly, this same type of reaction has been proposed for the role of Zn^{2+} ion in the NADH reduction of acetaldehyde, catalyzed by horse liver alcohol dehydrogenase (Richards, 1970). This reaction shows an isotope effect for hydride *vs.* deuteride transfer (k_H/k_D = 3.11) very similar to the results in the present study of glyoxalase-I (Mahler *et al.*, 1962). The possibility that the metal ion in glyoxalase-I functions as a superacid which polarizes the α-ketone group to such an extent that variation of the side chain of the α-ketoaldehydes does not make a significant additional contribution to the polarity of the α-ketone group is an attractive possibility. One problem, as Davis and Williams observed, is that the yeast enzyme binds its metal ion very tightly; attempts to remove and replace it with other cations have not been successful (Davis and Williams, 1966). Mammalian sources of glyoxalase-I bind their metal ions less tightly; they can be removed and replaced with other cations (Davis and Williams, 1966; Mannervik *et al.*, 1972). A detailed analysis of the role of the metal ion in glyoxalase-I from various sources is under way; preliminary comparative studies on yeast glyoxalase-I and several mammalian erythrocyte sources of glyoxalase-I suggest that the basic mechanisms of these enzymes are similar (Vander Jagt and Han, 1973). The erythrocyte sources have several isozymes of the enzyme (L. P. B. Han and D. L. Vander Jagt, manuscript in preparation).

The coenzyme role of glutathione appears mainly to be one of supplying groups for substrate binding rather than supplying catalytic groups. The pH rate studies indicate the involvement of dissociable groups with p*K* values of 4.7–5.2 and 8.4–8.9 which may be a carboxyl and an amino group on the enzyme geometrically arranged as points for ionic attraction of the α-amino group and one of the carboxyl groups of glutathione. The absence of any pH influence on V_{max} in the pH range studied indicates that no dissociable group important in the catalytic reaction was encountered in this range. This suggests that general acid–base mechanisms are not involved in the rate-determining step, which is consistent with a previous observation that, at pH 7, V_{max} for the glyoxalase-I reaction with methylglyoxal is identical in H_2O and D_2O (Vander Jagt and Han, 1972).

The use of pH–rate profiles with derivatives of glutathione should give some indication of the validity of the above suggestion that the observed p*K* values assigned to groups on the enzyme may be from dissociable groups to which glutathione is attracted by ionic interactions. Meaningful pH–rate studies with *N*-acetyl-GSH could not be carried out with the α-ketoaldehydes used in the present study owing to the experimental conditions. The availability of α-ketoaldehydes whose hemimercaptal with *N*-acetyl-GSH is active in the glyoxalase-I reaction and possesses a smaller K_M value may overcome these difficulties. If the conclusion that the specificity of the glyoxalase-I reaction resides primarily in the binding step, k_1, is correct, and if the value calculated for k_1 when phenylglyoxal was used is accurate, then there may well be α-ketoaldehydes whose hemimercaptals with glutathione and derivatives of glutathione will bind to glyoxalase-I at a much faster rate than was calculated for phenylglyoxal, which is at least 100-fold below a diffusion-controlled rate. We are continuing our examination of the specificity of glyoxalase-I.

References

Alexander, N. M. (1958), *Anal. Chem. 30*, 1292.

Bartfai, T., Ekwall, K., and Mannervik, B. (1973), *Biochemistry 12*, 387.

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

Davis, K. A., and Williams, G. R. (1966), *Biochim. Biophys. Acta 113*, 393.

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

Dixon, M., and Webb, E. C. (1964), Enzymes, 2nd ed, New York, N. Y., Academic Press.

Doering, W. von E., Taylor, T. I., and Schoenewaldt, E. F. (1948), *J. Amer. Chem. Soc. 70*, 455.

Franzen, V. (1956), *Chem. Ber. 89*, 1020.
Hammes, G. G., and Schimmel, P. R. (1970), *Enzymes, 3rd Ed., 2*, 67.
Hine, J., and Koser, G. F. (1971), *J. Org. Chem. 36*, 3591.
Kermack, W. D., and Matheson, N. A. (1957), *Biochem. J. 65*, 48.
Lineweaver, H., and Burk, D. (1934), *J. Amer. Chem. Soc. 56*, 658.
Mahler, H. R., Baker, R. H., Jr., and Shiner, V. J., Jr. (1962), *Biochemistry 1*, 47.
Mannervik, B., Lindstrom, L., and Bartfai, T. (1972), *Eur. J. Biochem. 29*, 276.
Martin, R. B., and Edsall, J. T. (1958), *Bull. Soc. Chim. Biol. 40*, 115.
Richards, J. H. (1970), *Enzymes, 3rd Ed., 2*, 321.
Rose, I. A. (1957), *Biochim. Biophys. Acta 25*, 214.
Utzinger, G. E., Strait, L. A., and Tuck, L. D. (1963), *Experientia 19*, 324.
Vander Jagt, D. L., and Han, L-P. B. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 3991.
Vander Jagt, D. L., and Han, L-P. B. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32*, 2560.
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.
Vander Jagt, D. L., Hansen, L. D., Lewis, E. A., and Han, L-P. B. (1972c), *Arch. Biochem. Biophys. 153*, 55.
Woodward, R. B., Wendler, N. L., and Brutschy, F. J. (1945), *J. Amer. Chem. Soc. 67*, 1425.

Conversion of Averufin into Aflatoxins by *Aspergillus parasiticus*†

M. T. Lin, D. P. H. Hsieh,* R. C. Yao, and J. A. Donkersloot

ABSTRACT: Resting mycelium of *Aspergillus parasiticus* ATCC 15517 efficiently converted averufin ($C_{20}H_{16}O_7$) into aflatoxin B_1, B_2, and G_1 with only minor dilution. Autoclaved mycelium did not catalyze this conversion. The removal of averufin from the medium and the incorporation into aflatoxin are both time dependent and detectable within 2 hr of incubation. [^{14}C]-Acetate-labeled averufin for the above experiments was prepared using a mutant of *A. parasiticus* which elaborates large amounts of averufin, but very little aflatoxin. Traces of averufin were also found in wild-type mycelium. The results indicate that the C_{20} polyketide-derived polyhydroxyanthraquinone averufin, or a metabolite closely related to it, is an intermediate in aflatoxin biosynthesis. A possible pathway for the conversion of averufin into aflatoxin B_1 is discussed.

The aflatoxins (Büchi and Rae, 1969; Figure 1) are a closely related group of secondary metabolites produced by certain *Aspergilli*. They can act as acute toxins and as chemical carcinogens in many animal species. They have been found as contaminants in various animal feeds and human foodstuffs, and have been implicated in the etiology of human liver cancer in certain parts of the world (Kraybill and Shapiro, 1969; Goldblatt, 1972). Two of the more readily noticeable acute *in vivo* effects of aflatoxin B_1 (or a metabolite derived from it; Moulé and Frayssinet, 1972), the most toxic and usually also the most prominent of the aflatoxins, are inhibition of DNA and DNA-directed RNA synthesis (reviewed by Wogan, 1969).

Biosynthesis of the aflatoxins has also attracted considerable attention (Mateles and Wogan, 1967; Lillehoj *et al.*, 1970; Detroy *et al.*, 1971). Incorporation experiments (Donkersloot *et al.*, 1968; Hsieh and Mateles, 1970), followed by degradative studies (Biollaz *et al.*, 1968a), have revealed that aflatoxin B_1 can be derived efficiently from acetate, and that methionine can act as a methyl donor for the methoxymethyl group (Figure 1). Based upon these findings, Biollaz *et al.* (1968b) proposed a scheme in which a C_{18} polyketide-derived polyhydroxynaphthacene would be an early intermediate. This compound would undergo endo oxygenation and yield, after rearrangement and isomerization, a difuroanthraquinone of the versicolorin series. Thomas (1965) had already speculated earlier that averufin or its derivative could be involved in aflatoxin biogenesis. Such an intermediate would, after oxidative removal of a quinone carbon as CO_2 and recyclization, yield a difuroxanthone of the sterigmatocystin type. The latter would subsequently undergo another loss of CO_2 and yield aflatoxin B_1 (reviewed by Biollaz *et al.*, 1970). This hypothesis was based, in part, upon the co-occurrence of aflatoxins and representatives of both the difuroanthraquinone series (versicolorin C; Heathcote and Dutton, 1969) and the difuroxanthone series (*O*-methylsterigmatocystin and aspertoxin; Burkhardt and Forgacs, 1968; Rodricks *et al.*, 1968; Waiss *et al.*, 1968) in certain members of the *Aspergillus flavus* group. Experimental support for a difuroxanthone precursor in aflatoxin biosynthesis was provided by Elsworthy *et al.* (1970), who showed that 5-hydroxydihydrosterigmatocystin could be incorporated into aflatoxins B_2 and G_2 (2% conversion) and by Hsieh *et al.* (1973), who showed that sterigmatocystin could be incorporated into aflatoxin B_1 (20% conversion). Experimental evidence for the early events in the biogenetic scheme proposed by Biollaz *et al.* (1970) has not been obtained so far.

To identify any early intermediates, a search was initiated for mutants blocked in aflatoxin synthesis. One such mutant produced large quantities of the polyhydroxyanthraquinone

† From the Department of Environmental Toxicology, University of California, Davis, California 95616, and the Section of Environmental Mechanisms, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014 (J. A. D.). *Received April 24, 1973.* A preliminary note on part of this work has appeared (Lin and Hsieh, 1973). This work was supported in part by Grants ES 00612 and ES 00125 from the U. S. Public Health Service.