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UNM - SANDIA CONTRACT 51-1985

"Chemical and Biological Properties of α -Dicarbonyls"

FINAL PROGRESS REPORT

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Summary:

The project "Chemical and Biological Properties of α -Dicarbonyls" supported during the past two years under Sandia Contracts 51-0060 and 51-1985 has been pursued in several areas.

1. The glyoxalase enzyme system which is able to disproportionate α -ketoaldehydes into α -hydroxycarboxylic acids has been studied extensively. The coenzyme for this system is a tripeptide, glutathione. One study on the ionization scheme of glutathione previously was approved for submission for publication in Biochem. Biophys. Research Communications. At the suggestion of the referees, this work was extended and then submitted as a full paper to Archives of Biochemistry and Biophysics. This paper has been tentatively accepted, pending some minor charges in the original manuscript. A copy of the revised manuscript is enclosed.
2. The range of α -ketoaldehydes disproportionated by the glyoxalase system has been evaluated for both aliphatic and aromatic compounds. A paper on the substrate specificity has been accepted for publication in Biochemistry. A copy of the manuscript is enclosed.
3. A model study of the glyoxalase reaction was studied in an attempt to obtain some additional information on the mechanism of the glyoxalase reaction. This work has been submitted to J. Org. Chem. for consideration. A copy of the manuscript submitted is enclosed.
4. An evaluation of the coenzyme role of glutathione and an evaluation of an unusual steric effect in the glyoxalase reaction are being studied at the present time. An abstract of some of this material has been published in Fed. Proc., 31, 3991 (1972). A copy of this abstract is enclosed.
5. Preliminary studies on the ability of α -ketoaldehydes to inhibit the growth of E. coli have been made. A summary of the initial results is enclosed.

Calorimetric Determination of the Micro
Ionization Constants of Glutathione¹

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ABSTRACT

The enthalpy changes for ionization of the four acidic protons of glutathione were measured calorimetrically, from both directions, using NaOH and HClO₄ as titrants. For the 3rd and 4th proton ionizations, $\Delta H = 7.56$ and 8.33 Kcal/mole, respectively. These are composite values for the overlapping ionizations of the SH and NH₃⁺ groups of glutathione. The micro-constants for ionization of the SH and NH₃⁺ groups were determined by resolving the composite enthalpy changes into the fractions proceeding by the sulfhydryl and amino routes. This calculation is based on the assignment of "standard" values to the enthalpy changes for ionization of the SH and NH₃⁺ groups. The micro-constants calculated are in good agreement with previously reported values obtained by different procedures. The ΔG° , ΔH° , and ΔS° values measured or calculated for all four ionization steps of glutathione are reported. The data suggest that glutathione has no unusual intramolecular interactions.

INTRODUCTION

The tripeptide glutathione (γ -L-glutamyl-L-cysteinylglycine) occurs widely in nature and its biological role is relatively broad. Potentiometric titration of glutathione (GSH) yields four pK values which sometimes are assigned directly to the two carboxyl, one amino, and one sulfhydryl groups. However, for GSH and related compounds, such as cysteine, the SH and NH_3^+ proton ionizations overlap, and, consequently, the pK values measured potentiometrically are composite values of the micro-constants, as shown in scheme 1 [1,2].

We wished to determine the micro-constants for GSH by a different procedure than those previously used, in order to have an independent evaluation of values already reported [1,2]. We report here our study of GSH using titration calorimetry. This method involves the calorimetric determination of the enthalpy changes for the ionization processes followed by calculation of the entropy changes from the enthalpy changes and free energy changes. These enthalpy and entropy change values can be used to obtain a value for K_2 (scheme 1) [3,4]. The assumption involved is that "standard" values of ΔH° or of ΔS° for ionization of the SH and NH_3^+ groups can be assigned. Since this assumption differs from those previously used to obtain micro-constants for GSH, an independent evaluation of the various methods is obtained.

scheme 1

Proton ionization scheme defining the microconstants for glutathione. Relationships of the microconstants to the macroconstants are:

- (1) $K_3 = K_S + K_N$
- (2) $1/K_4 = 1/K_{SN} + 1/K_{NS}$
- (3) $K_3 K_4 = K_S K_{SN} = K_N K_{NS}$

MATERIALS AND METHODS

A Tronac titration calorimeter, modified as previously described [5], was used for the heat measurements. The titration vessel initially contained 25.0 ml of 0.01 M glutathione in acid or base form which was then titrated with 0.7 M NaOH or 0.5 M HClO₄. A typical run consisted of 20 data points taken both in the fore and after periods and approximately 50 data points taken in the main period, all at 20 second intervals.³ A representative enthalpogram is shown in figure 1. All data were taken at $25.0 \pm 0.1^\circ\text{C}$.

All solutions were prepared using distilled, deionized, oxygen free water. Glutathione was obtained from Sigma Chemical Co. and was found to be at least 99% pure as determined both from the endpoints on the thermograms and from titration with N-ethylmaleimide [6].

The necessary pK values for the macro-ionization processes were obtained from the literature [2,7,8,9,10]. Where necessary, corrections for ionic strength effects were made using the Debye-Huckel equation with $a = 5 \text{ \AA}$ [11].⁴ The pK_a values used for the calculations in this study were 2.12, 3.73, 9.02, and 10.14 (at $\mu = 0$). The method of obtaining the enthalpy change values from the thermograms has been described [12,13]. Since the ionic strength was low, the measured enthalpy change, ΔH , was assumed to be equal to ΔH° . To simplify the calculations, the titration data were divided into two regions (i.e., carboxyl and thiol-amine regions), with the data in each region being treated as a dibasic acid.

The measured enthalpy changes corresponding to K₃ and K₄ can be

used independently to calculate the fractions proceeding by the sulfhydryl and amino routes by the use of equations 2 and 3, by assigning "standard" values to ΔH_S° , ΔH_N° , ΔH_{NS}° , and ΔH_{SN}° . (See scheme 1 for subscript notation.) An alternate method is to assign a "standard" value to either ΔH_N° or to ΔH_S° and combine equations 2 and 3 by assuming that $\Delta H_S^\circ = \Delta H_{NS}^\circ$ and that $\Delta H_{SN}^\circ = \Delta H_N^\circ$.

$$\Delta X_3^\circ = \alpha \Delta X_S^\circ + (1-\alpha) \Delta X_N^\circ \quad (2)$$

$$\Delta X_4^\circ = (1-\alpha) \Delta X_{NS}^\circ + \alpha \Delta X_{SN}^\circ \quad (3)$$

$$K_2 = \alpha / (1-\alpha) \quad (4)$$

α is the fraction of glutathione ionizing by the sulfhydryl route in the third stage and by the amino route in the fourth stage. $X = S$ or H

The two methods must ultimately give the same average value of K_2 , but they give different information concerning the dependence of K_2 on the "standard" values assigned and on the experimental data. Another method of calculation is to use the ΔS° values to calculate K_2 in the same manner as the ΔH° values.

A value of 6.2 ± 0.2 kcal/mole was assigned to ΔH_S° and ΔH_{NS}° . This is equal to the average of ΔH_S° values reported for thioglycolic acid, ethanethiol, β -acetylaminoethanethiol, 2-hydroxyethanethiol, β -mercaptopropionic acid, and thiosalicylic acid [14]. A value of 10.0 ± 0.5 kcal/mole corresponding to the average ΔH_N° values for aspartic acid, glutamic acid, glycine, S-methylcysteine, and glycylglycine was assigned to ΔH_N° and ΔH_{SN}° [14]. From a similar consideration of the entropy changes for ionization of α -amino acids and β -substituted ethanethiols, values of -8 ± 2 and -28 ± 8 cal/deg.-mole were assigned to

ΔS_N° and ΔS_{SN}° and to ΔS_S° and ΔS_{NS}° , respectively [14].

pKa values for cysteine ethyl ester (Sigma) were measured on a Radiometer Titrigraph, Type SBR2c employing a glass electrode, type G2222B. Corrections of pKa values to $\mu = 0$ were made as with GSH. The values used in this study were 6.45 and 8.99. That the higher pKa corresponds to ionization of the SH proton was established by following the ionization of the SH group spectrophotometrically at 240 nm as the pH was increased. Cysteine ethyl ester was also checked for purity by titration of the SH group with N-ethylmaleimide and was 799% pure by this procedure.

RESULTS

The enthalpy changes obtained for the four ionizations of GSH measured from both the acid and base directions are shown in Table I. Reactions 1-4 represent H^+ additions and reactions 5-8, H^+ removals. Adding reactions 1 and 5, 2 and 6, 3 and 7, or 4 and 8 (Table I) in each case gives the net reaction $H^+ + OH^- = H_2O$. The ΔH values for these sums are -13.49, -13.45, -13.37, and -13.36 kcal/mole respectively, in excellent agreement with the reported value for the heat of neutralization of water of -13.40 at $\mu = 0.04$. [15] Furthermore, this ionic strength (0.04) is close to the center of the ionic strength range used to obtain the heat data. (See Table I.) Enthalpy values show a small ionic strength dependence; both the magnitude and direction of deviation of the values from Table I compared to the accepted value of -13.40 kcal/mole are as expected, although all of the deviations are within our estimated uncertainties. The agreement among these numbers shows that the thermal stoichiometry for each step in the ionization scheme of GSH is consistent with the net removal of one proton and shows no evidence of any slow intramolecular reactions involving an enthalpy change not equal to zero.

The thermodynamic parameters calculated or measured for all four macro proton ionization steps are given in Table II.

Table III shows results obtained from the various combinations of experimental data, equations, and assumptions that can be used to obtain a K_2 value from the ΔH° and ΔS° values. The error limits given in Table III were calculated from equations 5 and 6 in accord with standard methods of calculating and combining errors.

$$\delta\alpha = \left[(\partial\alpha / \partial \Delta X_S^\circ)^2 (\partial \Delta X_S^\circ)^2 + (\partial\alpha / \partial \Delta X_N^\circ)^2 (\partial \Delta X_N^\circ)^2 + (\partial\alpha / \partial \Delta X_3^\circ)^2 (\partial \Delta X_3^\circ)^2 + (\partial\alpha / \partial \Delta X_4^\circ)^2 (\partial \Delta X_4^\circ)^2 \right]^{1/2} \quad (5)$$

$$\delta K_Z = \delta\alpha / (1-\alpha)^2 \quad (6)$$

The error limits obtained for K_Z in Table III are much smaller for the calculations using the combination of equations 2 and 3 than for the calculations using only one of these equations. However, the combination of equations 2 and 3 requires the assumptions that $\Delta X_S^\circ = \Delta X_{NS}^\circ$ and that $\Delta X_N^\circ = \Delta X_{SN}^\circ$, and the additional error from this source should be included. At this time, it is not possible to evaluate this error, but its magnitude is indicated by the difference between the K_Z values calculated independently from equations 2 and 3 where these assumptions need not be made. From these considerations we conclude from the ΔH° data that $K_Z = 1.5 \pm 0.6$ and from the ΔS° data that $K_Z = 0.8 \pm 0.3$ and that there probably is no significant difference between the K_Z values obtained from ΔH° and ΔS° data in this case. The well known sensitivity of ΔS° values to ionic charges which are not located at the reaction center makes K_Z values calculated from the entropy changes less reliable than the values calculated from the enthalpy changes; i.e., the assumptions that $\Delta S_{SN}^\circ = \Delta S_N^\circ$ and that $\Delta S_{NS}^\circ = \Delta S_S^\circ$ are less reliable than the assumptions that $\Delta H_{SN}^\circ = \Delta H_N^\circ$ and $\Delta H_{NS}^\circ = \Delta H_S^\circ$. Furthermore, there are large uncertainties in assigning the "standard" ΔS values. For these reasons the Discussion will consider only the enthalpy data. Table IV gives the micro pK values calculated using $K_Z = 1.5$.

The value of K_Z from equation 4 is very sensitive to the "standard"

ΔH values used to solve equations 2 and 3. However, since K_Z can be obtained both from K_3 and K_4 , a comparison of the two values obtained reflects on the two ΔH values assigned. If $\Delta H_S = 5.2$ Kcal/mole rather than the assigned 6.2 Kcal/mole, solution of equations 2 and 3 would give K_Z values of 1.0 and 1.9. If $\Delta H_N = 11.0$ Kcal/mole rather than the assigned 10.0 Kcal/mole, K_Z values of 2.6 and 0.8 would result. Consequently, the values in Table III using standard ΔH values of 6.2 and 10.0 Kcal/mole appear reasonable in light of the agreement between the K_Z values obtained for K_3 and K_4 .

To further support the assignment of "standard" ΔH values of 6.2 and 10.0 Kcal for the SH and NH_3^+ proton ionization of GSH, the enthalpy changes were measured for the base titration of the SH and NH_3^+ protons of cysteine ethyl ester. Cysteine, like glutathione, has overlapping SH and NH_3^+ proton ionizations [3]. However, esterification of the carboxyl group changes the pK's of the SH and NH_3^+ protons significantly so that they can be obtained by direct potentiometric titration. Consequently, they can also be titrated calorimetrically to obtain the enthalpies $\Delta H_{NH_3^+}$ and ΔH_{SH} directly. The data are in Table V. The heats for base titration are -3.33 and -7.17 Kcal/mole for the NH_3^+ and SH protons, respectively. Correction for the heat of neutralization of water (-13.40 Kcal/mole) gives values of 10.07 and 6.23 Kcal/mole, in excellent agreement with the "standard" values assigned to $\Delta H_{NH_3^+}$ and ΔH_{SH} .

DISCUSSION

Micro-ionization Constants of GSH:

For GSH and related compounds, such as cysteine, the SH and NH_3^+ proton ionizations overlap, and, consequently, the pKa values measured potentiometrically are composite values of the micro-constants, as shown in scheme 1. Several investigators have studied the ionization of GSH using different assumptions in order to solve the three equations of scheme 1 for the four micro-constants: Benesch and Benesch attempted to follow the sulfhydryl ionization spectrophotometrically, as has been done with cysteine [1]. However, GSH, unlike cysteine, does not exhibit an absorption maximum around 235 nm; an approximate value of K_2 about equal to unity was reported. In cases where the spectrophotometric method is used to follow the sulfhydryl ionization, one must assume that the molar absorptivities of the two forms $\text{R}-\text{S}^-$ and $\text{R}-\text{S}^-$ are the same. Other studies on the wavelength dependence of the apparent degree of sulfhydryl ionization in various thiol containing compounds suggests that this assumption is not valid [16,17].

Another procedure involves using model compounds in order to determine a value to be assigned to one of the micro-constants of GSH. Martin and Edsall [2] used the model compound S-methylglutathione to obtain a pK value for the NH_3^+ proton, which they then assigned to K_N . The assumption here is that this modification of the sulfhydryl group does not appreciably affect K_N and also that K_N in GSH is not sensitive to the degree of sulfhydryl ionization. These authors reported $K_2 = 1.8$.

The present study involves a method which makes quite different assumptions than those already mentioned. The assumption made

in this study is that the enthalpy (or entropy) change measured for a given proton ionization which is a composite of two ionizing groups, such as the SH and NH_3^+ groups of cysteine or GSH, can be resolved into the fractions proceeding by each route by assigning "standard" ΔH° (or ΔS°) values for SH and NH_3^+ protons. Inherent in this assumption is the requirement that the two groups have significantly different ΔH° (or ΔS°) values. Application of this procedure to cysteine has been reported previously [3]. We assigned ΔH° values of 6.2 and 10.0 Kcal/mole for the SH and NH_3^+ groups of GSH by taking the average of enthalpies reported for a number of compounds considered reasonable models for GSH. By assigning ΔH° values for both groups, K_2 can be evaluated independently from data for the 3rd and 4th ionization of GSH. Using this procedure, the heats of reaction for K_3 and K_4 (Table I) are resolved by equations 2 and 3 to give values for K_2 of 1.8 and 1.3, respectively. The average $K_2 = 1.5$ is in good agreement with Martin and Edsall's value of 1.8. It should be pointed out that this agreement is between experiments which make different assumptions. Martin and Edsall proposed a "standard" value for K_N which represents an assumption about the free energy change associated with this step. The present study makes an enthalpy assumption in assigning "standard" ΔH° values. The results of the present study confirm and extend the results of Martin and Edsall.

FOOTNOTES

1. This work was supported by the U.S. Atomic Energy Commission under Sandia Corporation Contract 51-0060, U.S. Public Health Service, National Institutes of Health (Grant 1 RO1 CA11850-01), The Petroleum Research Fund administered by the American Chemical Society, and a Public Health Service Research Career Development Award (5-K4-GM-42, 545,02) from National Institutes of Health to L.D.H.; a preliminary report of this work was presented at the 62nd annual meeting of the American Society of Biological Chemists in San Francisco, June, 1971; Fed. Proc. 30, 1252 (1971).
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3. See reference 5 for a definition of fore, after, and main periods.
4. Use of the Debye-Huckel equation with $a = 2 \text{ \AA}$ and $8 \text{ \AA}$ to calculate pK values at $\mu = 0$ was also carried out. Use of these values in the calculations (ref. 12,13) changed the enthalpies in TABLE I by less than 0.03 kcal/mole.

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TABLE I

Heats of Reaction of Glutathione in Dilute Aqueous Solution at 25°

Reaction ^a	ΔH (kcal/mole) ^b	Range of μ	No. of Runs
1. $H^+ + G^{-3} = HG^{-2}$	-8.37 ± 0.06	0.029 - 0.048	3
2. $H^+ + HG^{-2} = H_2G^-$	-7.58 ± 0.04	"	3
3. $H^+ + H_2G^- = H_3G$	-0.15 ± 0.09	0.027 - 0.031	3
4. $H^+ + H_3G = H_4G^+$	-0.43 ± 0.13	"	3
5. $OH^- + HG^{-2} = G^{-3} + H_2O$	-5.12 ± 0.07	0.026 - 0.063	4
6. $OH^- + H_2G^- = HG^{-2} + H_2O$	-5.87 ± 0.03	"	4
7. $OH^- + H_3G = H_2G^- + H_2O$	-13.22 ± 0.06	0.011 - 0.018	4
8. $OH^- + H_4G^+ = H_3G + H_2O$	-12.93 ± 0.11	"	4

a. First series of reactions (1-4) used 0.5139 M $HClO_4$ and second series (5-8) used 0.7042 M NaOH as titrant.

b. $\pm$ Values are the standard deviations of the means between runs.

TABLE II

Thermodynamic Constants for Proton Ionization from Glutathione in
Dilute Aqueous Solution at 25°.

Ionization	<u>1st</u>	<u>2nd</u>	<u>3rd</u>	<u>4th</u>
Macro-pKa ($\mu = 0.0$) ^a	2.12	3.73	9.02	10.14
ΔG° (Kcal/mole)	+2.88 $\pm$ 0.06	+5.07 $\pm$ 0.06	+12.26 $\pm$ 0.04	+13.78 $\pm$ 0.04
ΔH° (Kcal/mole) ^b	+0.45 $\pm$ 0.13	+0.17 $\pm$ 0.09	+7.56 $\pm$ 0.06	+8.33 $\pm$ 0.06
ΔS° (cal/deg-mole)	-8.2 $\pm$ 0.6	-16.5 $\pm$ 0.5	-15.9 $\pm$ 0.3	-18.5 $\pm$ 0.3

a. From text refs. 3b, 10, 15, 16, 17.

b. $\pm$ Values are the estimated uncertainties.

TABLE III

Values of K_z , α , and Errors in K_z and α Obtained from Various Computational Methods

Equations ^a Used	Experimental Data Used	Assumptions Used	α	$\delta\alpha^b$	K_z	δK_z^c
2	ΔH°_3	$\Delta H^\circ_S = 6.2, \Delta H^\circ_N = 10.0$	0.64	± 0.06	1.8	± 0.5
3	ΔH°_4	$\Delta H^\circ_{NS} = 6.2, \Delta H^\circ_{SN} = 10.0$	0.56	± 0.11	1.3	± 0.6
2 and 3	ΔH°_3 and ΔH°_4	$\Delta H^\circ_N = 10.0, \Delta H^\circ_{SN} = \Delta H^\circ_N,$ $\Delta H^\circ_{NS} = \Delta H^\circ_S$	0.59	± 0.02	1.5	± 0.1
2 and 3	ΔH°_3 and ΔH°_4	$\Delta H^\circ_S = 6.2, \Delta H^\circ_{SN} = \Delta H^\circ_N,$ $\Delta H^\circ_{NS} = \Delta H^\circ_S$	0.61	± 0.02	1.6	± 0.1
Average					1.5	$\pm 0.6^d$
2	ΔS°_3	$\Delta S^\circ_S = -28, \Delta S^\circ_N = -8$	0.40	± 0.17	0.7	± 0.5
3	ΔS°_4	$\Delta S^\circ_{NS} = -28, \Delta S^\circ_{SN} = -8$	0.48	± 0.59	0.9	± 2.2
2 and 3	ΔS°_3 and ΔS°_4	$\Delta S^\circ_N = -8, \Delta S^\circ_{SN} = \Delta S^\circ_N,$ $\Delta S^\circ_{NS} = \Delta S^\circ_S$	0.43	± 0.02	0.8	± 0.1
2 and 3	ΔS°_3 and ΔS°_4	$\Delta S^\circ_S = -28, \Delta S^\circ_{SN} = S^\circ_N,$ $\Delta S^\circ_{NS} = \Delta S^\circ_S$	0.44	± 0.04	0.8	± 0.1
Average					0.8	$\pm 0.3^d$

a. Numbers indicate equation numbers in the text.

b. Calculated from equation 5 in the text.

c. Calculated from equation 6 in the text.

d. See text for explanation of how final value of K_z was obtained.

TABLE IV

Micro pKa values^a for GSH, $\mu = 0$, 25°

$$K_Z = 1.5$$

$$pK_S = 9.24$$

$$pK_N = 9.42$$

$$pK_{SN} = 9.92$$

$$pK_{NS} = 9.74$$

a. See scheme 1 for reference to subscript notation.

TABLE V

Heats of Reaction of Cysteine ethyl ester in Dilute Aqueous Solution at 25°

<u>Reaction</u> ^a	<u>ΔH (kcal/mole)</u> ^b	<u>Range of μ</u>	<u>No. of Runs</u>
1. $\text{OH}^- + \text{H}_2\text{C}^+ = \text{HC} + \text{H}_2\text{O}$	-3.33 ± 0.03	0.0098-0.010	3
2. $\text{OH}^- + \text{HC} = \text{C}^- + \text{H}_2\text{O}$	-7.17 ± 0.01	0.010-0.013	3

a. 0.7042 M NaOH used as titrant.

b. $\pm$ values are the standard deviations of the means between runs.

Legend for Figure 1

Representative enthalpogram showing a computer plot of the results of a calorimetric titration of glutathione using HClO_4 as titrant. Enthalpies corresponding to K_3 and K_4 are obtained from the points between A and B and enthalpy changes for ionization of the carboxyl groups are obtained from the points between B and C. (See ref. 16,17 for calculations method.)

Kinetic Evaluation of Substrate Specificity in the
Glyoxalase-I Catalyzed Disproportionation of α -Ketoaldehydes*

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¹ Details will be reported elsewhere in a paper on some chemical reactions of α -ketoaldehydes.

Abstract:

The dissociation constants, K_{diss} , of the adducts formed in the pre-enzymic reaction between substrates (α -ketoaldehydes) and coenzyme (glutathione, GSH) in the glyoxalase system have been determined for methylglyoxal, phenylglyoxal, and a series of meta or para substituted phenylglyoxals including p -CH₃, p -OCH₃, m -OCH₃, p -Br, p -Cl, p -OH, p -NO₂, and p -phenyl. For methylglyoxal, $K_{diss} = 3.0 \pm 0.5 \times 10^{-3}$ M in the pH range 5-9; for phenylglyoxal, $K_{diss} = 0.60 \pm 0.05 \times 10^{-3}$ M, increasing somewhat at the higher end of this pH range. At pH 7, all the substituted phenylglyoxals show K_{diss} ca. $1-3 \times 10^{-3}$ M. The lack of substituent effects on K_{diss} is reflected also in the similar rates of adduct formation, followed at pH 3. The consistent values observed for K_{diss} when the initial GSH and α -ketoaldehyde concentrations are varied suggest only 1:1 adducts are formed. The data support the idea that the adducts are hemimercaptals. Glyoxalase-I shows very broad specificity in the disproportionation of these adducts into the corresponding GSH thiol esters of the α -hydroxy-carboxylic acids. In every case the hemimercaptal is the glyoxalase-I substrate; and all V_{MAX} values are within a factor of 4 of V_{MAX} for the methylglyoxal-GSH adduct, again showing complete insensitivity to ring substituents. However, the K_M values for the substituted phenylglyoxals-GSH adducts (2×10^{-4} to 2×10^{-5} M) are smaller than K_M for the methylglyoxal-GSH adduct (3×10^{-4} M). The glyoxalase-I reaction is inhibited

competitively by free GSH, $K_i = 5 \times 10^{-3}$ M. Although the glyoxalase-I reaction is known to involve intramolecular hydride transfer, the marked insensitivity of the reaction to variations in the α -ketoaldehydes suggests that hydride transfer may not be the rate determining step.

The disproportionation of α -ketoaldehydes into the corresponding α -hydroxycarboxylic acids, catalyzed by the glyoxalase system, appears to be a common intracellular reaction (Dakin and Dudley, 1913; Neuberger, 1913; Lohmann, 1932; Knox, 1960) and is of interest for several reasons: (a) the biological importance of the glyoxalase system is still unclear, although a number of roles have been suggested such as a role in protection against α -ketoaldehyde toxicity (Salem, 1954), and a role in regulation of cellular growth (Egyud and Szent-Gyorgyi, 1966; Szent-Gyorgyi et al., 1967; Szent-Gyorgyi, 1968); (b) the reaction utilizes glutathione (GSH) as coenzyme, one of many roles for GSH; and (c) the reaction mechanism is unusual in that GSH and α -ketoaldehydes first combine in a non-enzymic reaction to form adducts which are the actual glyoxalase substrates (Cliffe and Waley, 1961; Davis and Williams, 1969). The reactions of the glyoxalase system proposed by Cliffe and Waley (1961) based upon a study using methylglyoxal as substrate are summarized in Scheme 1.

To date, very little has been reported on the range of α -ketoaldehydes which are substrates for glyoxalase-I (S-lactoyl-glutathione methylglyoxal-lyase (isomerizing, EC 4.4.1.5), and only methylglyoxal, the α -ketoaldehyde commonly found intracellularly, has been analyzed in detail as a substrate for glyoxalase-I

(referred to hereafter simply as glyoxalase). The present study was initiated to answer the following: (a) is the pre-enzymic reaction between GSH and α -ketoaldehyde consistent with hemi-mercaptal formation; (b) what is the substrate specificity for glyoxalase in terms of variation of the α -ketoaldehyde; and (c) is the actual glyoxalase substrate the α -ketoaldehyde-GSH adduct, as reported for methylglyoxal. We report here our results from a study of methylglyoxal and a series of substituted phenylglyoxals as glyoxalase substrates (reaction 1-3, Scheme 1).

Scheme 1

GSH = γ -L-glutamyl-L-cysteinylglycine

Experimental Section

Materials:

GSH (Sigma) was found to be >99% pure by sulfhydryl titration with N-ethylmaleimide (Alexander, 1958). Yeast glyoxalase-I (Sigma) was obtained as a 50% glycerol solution. Commercial methylglyoxal, 40% aqueous solution (Aldrich) was purified by distillation (Kermack and Matheson, 1957). Standardization of methylglyoxal solutions was carried out by preparation of the bis-2,4-dinitrophenylhydrazone derivative which in basic solution showed λ_{MAX} 555 nm, molar extinction coefficient (ϵ) $4.14 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. Another method involved preparation of the bis-semicarbazone derivative which at pH 7 showed λ_{MAX} 283 nm, $\epsilon 2.55 \times 10^4$ (lit. λ_{MAX} 286, $\epsilon 3.20 \times 10^4$; Alexander and Boyer, 1971); the two methods gave good agreement. A series of meta or para substituted phenylglyoxals including H, p-CH₃, p-OCH₃, p-Cl, p-Br, p-phenyl, m-OCH₃, and p-NO₂ was prepared either by selenium dioxide oxidation of the corresponding acetophenones (Riley and Gray, 1943) or by use of Kornblum's procedure (1966) involving conversion of the substituted phenacyl bromide into the nitrate ester with silver nitrate, followed by treatment with sodium acetate in dimethylsulfoxide to convert the nitrate ester into the substituted phenylglyoxal. Para-hydroxyphenylglyoxal was prepared by oxidation of p-benzoylacetophenone, followed by hydrolysis. The products were generally isolated in their hydrated form; structures were confirmed by evaluation of the i.r., u.v., and n.m.r. spectra as well as elemental analysis of the dioxime

derivatives. The u.v. spectral data of the substituted phenylglyoxal hydrates are given in TABLE I.

Methods:

Dissociation Constants: The dissociation constants of the adducts of GSH and α -ketoaldehyde are defined as

$$K_{\text{diss}} = \frac{[\text{total } \alpha\text{-ketoaldehyde}][\text{GSH}]}{[\text{adduct}]}$$

where total α -ketoaldehyde at equilibrium is essentially equal to the concentration of the hydrated form, owing to the high degree of hydration of these very reactive aldehydes. The concentrations at equilibrium can be obtained from the absorbance at some wavelength λ if one knows the molar extinction coefficients, ϵ .

For the reaction between methylglyoxal and GSH, K_{diss} was determined at 240 nm, 25°, in the pH range 5-9. For methylglyoxal, $\epsilon_{\text{MG}}^{4M^{-1}cm^{-1}}$ throughout this pH range (lit. ϵ_{MG} 5; Racker, 1951); for GSH, ϵ_{GSH} 61, 61, 65, 115, 209, 834, 2280 at pH 5.25, 5.74, 6.18, 6.85, 7.47, 8.26 and 8.98, respectively, all at 240 nm. By using very high or very low $[\text{GSH}]/[\text{methylglyoxal}]$ to push adduct formation toward completion, ϵ_{AD} was estimated to be 440, pH independent (lit. ϵ_{AD} 395, Davis and Williams, 1969; ϵ_{AD} 220, Cliffe and Waley, 1961). In general, at each pH value the determination of K_{diss} was made at 5-10 different concentrations of GSH and methylglyoxal.

For the reaction of GSH with the series of phenylglyoxals, K_{diss} values could be determined at wavelengths where GSH does not absorb. The phenylglyoxal adducts were evaluated over a range of pH,

similar to the methylglyoxal study. The dissociation constants for substituted phenylglyoxal adducts were evaluated at pH 7. The λ and ϵ values used are summarized in TABLE I. Absorbance measurements for determinations of K_{diss} were obtained either with a Cary 15 or with a Gilford 222 modified Beckmann DU. Both spectrophotometers were temperature controlled with circulating water baths.

Kinetics: Reaction rates for adduct formation were monitored with the spectrophotometers mentioned above. All rate constants were from computer calculated least squares lines of first order plots; correlation coefficients were generally better than 0.999.

The glyoxalase catalyzed disproportionation of the GSH adduct of methylglyoxal was followed at 240 nm by monitoring the appearance of thiolester product, ϵ_p 3300 M⁻¹cm⁻¹ (lit. ϵ 3370, Racker, 1951; ϵ 3300, Cliffe and Waley, 1961). Initial rates were obtained from the initial changes in absorbance as $\frac{dP}{dt} = \frac{\Delta \text{Abs.}}{\Delta t(\epsilon_p - \epsilon_{AD})}$. From the initial concentrations and K_{diss} value, the adduct concentrations could be calculated for each initial rate. The data were treated by the Michaelis Menten scheme (1913) and V_{MAX} and K_M values obtained from double reciprocal plots (Lineweaver and Burk, 1934). Methylglyoxal also was used to standardize solutions of glyoxalase. Sufficient concentrations of the GSH adduct of methylglyoxal can be obtained to saturate glyoxalase at concentrations used in the initial rate studies. The V_{MAX} values from saturation studies agree very well with V_{MAX} values obtained from the

double reciprocal plots. Commercial yeast glyoxalase, specific activity 200-600 $\mu\text{moles/min/mg.}$, was generally diluted 250 fold in pH 7.0 phosphate buffer containing 100 $\mu\text{g/ml.}$ bovine serum albumin. In all initial rate studies and in the standardization of the glyoxalase stock solution, 20 $\mu\text{l.}$ enzyme solution/3ml. reactant solution was used. This amount of enzyme gave convenient initial rates to follow and was in the range where the initial rate is proportional to glyoxalase concentration. At higher enzyme concentrations, the pre-enzymic reaction to form adduct is rate limiting (Cliffe and Waley, 1961).

The initial rates for disproportionation of the GSH adducts of substituted phenylglyoxals were followed at the apparent isosbestic points of the substituted phenylglyoxal hydrates and their GSH adducts. Representative spectra for phenylglyoxal are shown in FIGURES 1 and 2 which summarize the general approach used in studying all of the substituted phenylglyoxals. In all cases, the thiol ester products have significantly lower molar extinction coefficients at the isosbestic points (λ_{iso}) than do the reactants. The reaction scheme for kinetic analysis of the substituted phenylglyoxals is as follows:

Initial rates, $\frac{dP}{dt} = \frac{\Delta \text{Abs}_{\lambda_{\text{iso}}}}{\Delta t (\epsilon_{\text{iso}} - \epsilon_p)}$ where $\Delta \text{Abs}_{\lambda_{\text{iso}}}$ is the

initial change in absorbance at the isosbestic point in time Δt , and $(\epsilon_{\text{iso}} - \epsilon_p)$ is the difference in molar extinction coefficients of reactant (S) and thiolester product (P). Owing to the rapidity of the pre-enzymic reactions (1 and 2 of Scheme 1), use of any wavelength other than λ_{iso} makes it difficult to convert initial changes in absorbance into initial rates unless one has knowledge of the rate constants k_1 - k_4 of Scheme 1. The λ_{iso} and ϵ values are given in TABLE I.

Kinetics of Product Hydrolysis: The thiol ester products were hydrolyzed at pH 10.5 using a Radiometer SBR2c Titrigraph pH-stat. The esters were prepared by running the enzyme reaction in neutral, unbuffered water.

Results

The dissociation constants for the GSH adducts of methylglyoxal and phenylglyoxal were determined in the pH range 5-9 for various initial concentrations of GSH and α -ketoaldehyde (TABLE II). For the methylglyoxal adduct, $K_{\text{diss}} = 3.0 \pm 0.5 \times 10^{-3}$ M throughout the pH range studied. The adduct of phenylglyoxal shows $K_{\text{diss}} = 0.60 \pm 0.05 \times 10^{-3}$ M from pH 5 - 7.5 and somewhat larger values as the pH increases. In both cases, the reasonably consistent values obtained at a given pH when the initial concentrations of both GSH and α -ketoaldehyde are varied indicate that only 1:1 adducts are formed.

The dissociation constants for GSH adducts of the substituted phenylglyoxals, determined at pH 7, are shown in TABLE II. The data again show that only 1:1 adducts are formed. The effects of substituents on K_{diss} are quite small, suggesting that the rates of adduct formation might also be insensitive to substituents. Cliffe and Waley (1961) reported that the rate of adduct formation between GSH and methylglyoxal depends only upon the methylglyoxal concentration. These authors suggested that dehydration of methylglyoxal (k_1 , Scheme 1) is rate determining in adduct formation. Thiol additions to reactive aldehydes such as formaldehyde also involve rate determining dehydration (Lienhard and Jencks, 1966;

Kallen and Jencks, 1966). In TABLE III are shown some apparent first order rate constants for approach to equilibrium, measured at pH 3, for the reaction of GSH with methylglyoxal and the series of phenylglyoxals. The reaction conditions were similar for each reaction. The effects of substituents on the rates are minor, in agreement with their small effects on K_{diss} . These rate constants are composites of the rate constants for the adduct forming reactions (reaction 1 and 2, Scheme 1) and cannot be assigned directly to the dehydration step k_1 . However, these apparent rate constants should parallel k_1 , suggesting that dehydration proceeds at a similar rate for all of the α -ketoaldehydes studied. A possible explanation for this insensitivity was obtained by analysis of the infrared spectra of the unhydrated α -ketoaldehydes. Solutions of substituted phenylglyoxals in acetonitrile were warmed over molecular sieves to prepare the unhydrated α -ketoaldehydes. Infrared spectra showed that the carbonyl stretching frequency for the aldehyde carbonyl was essentially constant at $1727 \pm 2 \text{ cm}^{-1}$, totally insensitive to substituent; the ketone carbonyl stretching frequencies were very sensitive to substituents¹. If one assumes that the energy of the carbonyl stretching frequency reflects sensitivity to nucleophilic attack, then the constant value observed in the aldehyde carbonyl frequencies is consistent with the insensitivity to substituents

observed in K_{diss} and in the rates of adduct formation, and it is consistent with the conclusion that the 1:1 adducts are hemimercaptals resulting from GSH addition only to the aldehyde carbonyl.

That the hemimercaptals are the actual glyoxalase substrates, as suggested by Cliffe and Waley (1961) for methylglyoxal, was confirmed by measuring initial rates of disproportionation of the GSH-methylglyoxal adduct under a wide range of different initial concentrations of GSH and methylglyoxal. Glyoxalase solutions generally were standardized under conditions of high initial methylglyoxal/GSH where the amount of free GSH at equilibrium is very small. Under these conditions the glyoxalase reaction proceeds very well. However, the reverse is not true; high initial GSH/methylglyoxal conditions give inconsistent initial rates. The presence of free GSH inhibits the glyoxalase reaction (Kermack and Matheson, 1957). Determination of the enzyme-inhibitor dissociation constant, K_i , and type of inhibition by direct evaluation of double reciprocal plots is difficult because GSH is involved in the pre-enzymic equilibrium reaction. Therefore, K_i was determined by working under saturation conditions. Various methylglyoxal/GSH ratios were used to form the adduct where in each case the concentration of adduct was sufficient to saturate the enzyme. V_{MAX} could be measured directly if the concentration of free GSH at equilibrium was low. At that ratio where the observed rate is $\frac{V_{MAX}}{2}$, $K_i = \frac{K_m [GSH]}{[adduct]}$. This ratio of $[GSH] / [adduct]$ is about 16. For the methylglyoxal adduct, $K_m = 3 \times 10^{-4}M$,

obtained from double reciprocal plots in runs where at each point the concentration of adduct exceeded the concentration of free GSH. The value of K_i for GSH inhibition is therefore about $5 \times 10^{-3} M$, and the inhibition must be competitive since high adduct concentrations overcome the inhibition.

Analysis of the adducts of the substituted phenylglyoxals as substrates demonstrated very broad specificity in the glyoxalase reaction. All of the GSH adducts of the substituted phenylglyoxals were comparable to the GSH adduct of methylglyoxal as substrates. Relative to the V_{MAX} for methylglyoxal, all α -ketoaldehydes examined fall within a 4-fold range, reflecting almost complete insensitivity to substituents. Results are shown in TABLE IV. The K_M values are smaller than K_M for the methylglyoxal adduct, ranging down to $2 \times 10^{-5} M$ for *p*-phenylphenylglyoxal. Consequently, many of the substituted phenylglyoxals are better substrates than methylglyoxal if one considers both binding to the enzyme (reflected in K_M) and the catalytic rate constant (reflected in V_{MAX}). Although many of the substituted phenylglyoxals show limited solubilities, in each case the initial rates could be determined at adduct concentrations which span K_M for that particular substrate.

To test whether the commercial enzyme preparation used in this study contained one or several glyoxalase species, preparations of different specific activity to methylglyoxal were tested with several different α -ketoaldehydes. In no case did the relative specificities

for several substrates show any variation from one preparation to another. Furthermore, commercial yeast glyoxalase preparations are available with specific activities within a factor of 2 of the highest activity reported for any yeast glyoxalase preparation (Davis and Williams, 1966). For these reasons, it was concluded that the broad specificity observed in the glyoxalase reaction probably represents the specificity of a single enzyme species.

In addition to the spectral changes which occur during the disproportionation reactions, and the observed broad tailing absorption around 240 m μ (FIGURE 2) characteristic of thiol esters (Racker, 1951), products were checked by hydrolysis of the thiol esters at pH 10.5. The rates were quite similar as expected for these compounds because the variations in the side groups are quite far removed from the ester linkages.

Discussion

The glyoxalase system consists of two enzymes, glyoxalase-I (S-lactoyl-glutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5) and glyoxalase-II (S-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6). The present study on the substrate requirements for glyoxalase-I supports the conclusion of Davis and Williams (1969) that glyoxalase-I is an oxidoreductive isomerase rather than a lyase, owing to the fact that the enzyme catalyzes the disproportionation of the hemimercaptals rather than using both GSH and the α -ketoaldehydes as substrates. The enzyme was named on the basis of the only well known intracellular α -ketoaldehyde, namely, methylglyoxal. In view of the broad specificity of glyoxalase-I and in view of reports of other intracellular α -ketoaldehydes (Sparkes and Kenny, 1969), which may very well be substrates for the enzyme, the official name for glyoxalase-I may be doubly in error.

Although methylglyoxal, phenylglyoxal, glyoxal, and hydroxypyruvaldehyde have been reported to be substrates for glyoxalase-I (Racker, 1952; Hopkins and Morgan, 1948), only methylglyoxal has been studied in any detail. Early estimates of the extent of adduct formation between GSH and methylglyoxal (Platt and Schroeder, 1934) gave values for K_{diss} ca. 10^{-2} M; this pro-

cedure involved rapid titration of the sulfhydryl groups in an equilibrium mixture of GSH, adduct, and methylglyoxal. Kermack and Matheson (1957) determined K_{diss} spectrophotometrically at 240 nm, pH 6.6, and reported a value of 5×10^{-3} M. Cliffe and Waley (1961) used the same procedure and reported a value of 2×10^{-3} M. These authors also estimated K_{diss} from their kinetic data in studies of glyoxalase-I and reported values $1-2 \times 10^{-3}$ M, increasing somewhat as the initial concentrations of GSH and methylglyoxal were lowered; they offered no explanation for this observation. These variations in reported values for K_{diss} of the GSH-methylglyoxal adduct raised the possibility that other than 1:1 adducts form, in which case the species distribution at equilibrium would depend both upon the initial GSH/methylglyoxal ratio and upon their actual concentrations. The results of the present study indicate that for all of the α -ketoaldehydes examined, only 1:1 hemimercaptals form to any significant extent.

The very broad substrate specificity shown by glyoxalase-I and the absence of any significant substituent effects in the disproportionation of the substituted phenylglyoxals raise questions about the mechanism of the reaction. It has been well established (Franzen, 1956; Rose, 1957) that the GSH-methylglyoxal adduct is disproportionated without loss of the alde-

hydric hydrogen (eq. 6), presumably by

open. The broad specificity of the enzyme for many α -ketoaldehydes is consistent with a detoxification role. However, if regulation of cellular growth involves intracellular α -ketoaldehydes presently unrecognized, these are likely to be substrates for glyoxalase.

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TABLE I

U.V. Data for Substituted Phenylglyoxal Hydrates, Their Adducts with Glutathione, and the Thiol Ester Products of the Glyoxalase Reaction^a

Substituent	λ_{MAX}	ϵ^b	λ_{Kdiss}	ϵ_{AD}	ϵ_{xPG}^c	λ_{iso}	ϵ_{iso}	ϵ_p^d
H	251	10,300	280	2450	1280	263	6,790	1,100
p-CH ₃	263	14,200	320	1250	165	273	10,300	1,010
p-OCH ₃	287	15,200	320	4190	1370	296	12,380	970
p-Br	264	14,800	310	1955	280	272	11,500	1,860
p-Cl	260	14,100	320	1350	380	270	10,000	1,020
p-phenyl	292	18,000	340	3180	795	303	16,000	2,280
m-OCH ₃	255	7,680	310	2060	2550	263	5,230	1,670
p-NO ₂	268	13,000	340	1190	575	270	13,000	10,700
p-OH	284	12,600	320	5735	4990	297	9,930	1,140

a Spectra recorded at 25 °C, phosphate buffer, pH 7.0, $\mu = 0.2$, half of ionic strength from buffer and half from added KCl.

b ϵ is the molar extinction coefficient ($M^{-1}cm^{-1}$) at the wavelength for maximum absorption, λ_{MAX} (nm), for the substituted phenylglyoxal hydrates.

c ϵ_{AD} and ϵ_{xPG} are the molar extinction coefficients for the GSH adducts and the substituted phenylglyoxals at the wavelengths (λ_{Kdiss}) used to determine the dissociation constants.

d ϵ_{iso} is the molar extinction coefficient at the isosbestic wavelength, λ_{iso} . ϵ_p is the molar extinction coefficient of the thiol ester product of the glyoxalase reaction at the isosbestic wavelength.

TABLE II

Dissociation Constants of the GSH Adducts of Substituted Phenylglyoxals, pH 7.0^a

<u>Substituent</u>	<u>K_{diss} (mM)</u>
H	0.60 $\pm$ 0.05
p-CH ₃	1.1 $\pm$ 0.1
p-OCH ₃	1.4 $\pm$ 0.1
p-Br	1.5 $\pm$ 0.2
p-Cl	3.6 $\pm$ 0.5
p-phenyl	0.89 $\pm$ 0.05
m-OCH ₃	1.2 $\pm$ 0.1
p-NO ₂	1.2 $\pm$ 0.2
p-OH	0.93 $\pm$ 0.05
methylglyoxal	3.0 $\pm$ 0.5

^a 25 °C, phosphate buffer, μ = 0.2, half of ionic strength from buffer and half from added KCl.

TABLE III

Apparent First Order Rate Constants for the Reaction of GSH
with α -Ketoaldehydes, pH 3.0^a

<u>substituted phenylglyoxal</u> ^b	<u>k (10⁻³ sec⁻¹)</u>
H	7.22 [±] 0.06
p-CH ₃	6.93 [±] 0.03
p-OCH ₃	7.95 [±] 0.05
p-Br	8.31 [±] 0.03
p-Cl	7.60 [±] 0.19
p-phenyl	8.04 [±] 0.05
m-OCH ₃	7.72 [±] 0.09
p-NO ₂	5.53 [±] 0.28
p-OH	6.35 [±] 0.11
methylglyoxal	6.55 [±] 0.41

^a formate buffer, 25 °C, μ = 0.2, half of ionic strength from buffer and half from added KCl.

^b [α -ketoaldehyde] = 0.3 - 0.4 mM; [GSH] = 5 mM;

TABLE IV

Kinetic Parameters K_M and V_{MAX} for the Glyoxalase-I.
Disproportionation of GSH Adducts of Methylglyoxal and
Substituted Phenylglyoxals.

<u>α-ketoaldehyde</u>	<u>K_M (M)</u>	<u>V_{MAX} (relative)^a</u>
methylglyoxal	3×10^{-4}	1.00
substituted phenylglyoxal		
H	2×10^{-4}	0.93
p-CH ₃	4×10^{-5}	0.27
p-OCH ₃	4×10^{-5}	0.24
p-Br	3×10^{-5}	0.46
p-Cl	2×10^{-5}	0.45
p-phenyl	2×10^{-5}	0.30
m-OCH ₃	6×10^{-5}	0.88
p-NO ₂	9×10^{-5}	0.72
p-OH	7×10^{-5}	0.31

^a V_{MAX} values all relative to methylglyoxal; all data at pH 7.0, 25 °C, phosphate buffer, $\mu = 0.2$, half of ionic strength from buffer and half from added KCl.

FIGURE LEGENDS

FIGURE 1. Spectrum of a 10^{-4} M solution of phenylglyoxal (Curve A), pH 7, 25 °C, and spectra of equilibrium mixtures of 10^{-4} M phenylglyoxal and added glutathione, 2.3×10^{-4} M (B), 6.9×10^{-4} M (C), and 1.2×10^{-3} M (D). K_{diss} was determined from the changes in absorbance at 280 nm. The glyoxalase-I catalyzed disproportionation of phenylglyoxal was monitored at the apparent isosbestic point, 263 nm. This general procedure was used for all of the substituted phenylglyoxals.

FIGURE 2. Spectrum of the thiol ester product of the glyoxalase-I catalyzed disproportionation of the GSH adduct of phenylglyoxal (Curve A); concentration of thiol ester 10^{-4} M. Spectrum B is for a 2×10^{-4} M solution of mandelic acid. Spectrum C is for a 2.3×10^{-4} M solution of glutathione. All spectra at 25°, pH 7.

FIGURE 2

Effects of Substituents on the Rates of Disproportionation of
Substituted Phenylglyoxals in Alkaline Solution^{1a,b}

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Abstract

A series of meta or para substituted phenylglyoxals, including H, p-CH₃, p-OCH₃, p-Br, p-Cl, p-phenyl, m-OCH₃, p-NO₂ and p-OH, were examined for Linear Free Energy Relationships between chemical reactivity and substituent constants, and between chemical reactivity and carbonyl stretching frequencies of the ketone and aldehyde carbonyls. At pH 12, the hydroxide ion catalyzed disproportionation of the phenylglyoxals into the corresponding mandelic acids follows the Hammett relationship with $\rho = 2.0$, indicative of a transition state stabilized by electron withdrawing groups. These rates of disproportionation also correlate quite well with the carbonyl stretching frequencies of the ketone carbonyls, both for the hydrated and the anhydrous phenylglyoxals. The aldehyde carbonyl stretching frequencies are essentially independent of ring substituents, $\nu_{\text{C=O}} = 1727 \pm 2 \text{ cm}^{-1}$. The disproportionation of α -ketoaldehydes is known to involve intramolecular hydride migration. The results of the present study suggest that hydride migration is the rate determining step in the disproportionation of this series of substituted phenylglyoxals.

Introduction

The glyoxalase system is composed of two enzymes, glyoxalase-I which utilizes glutathione (GSH) as coenzyme and catalyzes the disproportionation of methylglyoxal into the thiol ester of lactic acid and GSH, and glyoxalase-II which hydrolyzes this thiol ester to regenerate GSH and liberate lactic acid.^{2,3} Scheme 1 summarizes the reactions of the glyoxalase system. Reactions 1 and 2 of Scheme 1 are pre-enzymic reactions to form a hemimercaptal which is the actual enzyme substrate^{4,5}. The net reaction in the glyoxalase system is the conversion of an α -ketoaldehyde into an α -hydroxycarboxylic acid. This is analogous to an intramolecular Cannizzaro reaction involving hydride migration from the aldehydic group to the α -carbon. The glyoxalase-I reaction (reaction 3 of Scheme 1) is known to occur without solvent exchange of the aldehydic hydrogen^{6,7}, as in the Cannizzaro reaction. The importance of the glyoxalase system is not yet clear. It is ubiquitous in nature, and there have been suggestions that the system may play an important role in the regulation of cell growth⁸. The general ability of methylglyoxal and other α -ketoaldehydes to inhibit the growth of both bacteria and mammalian cells is well established^{9,10} and has resulted in the specific suggestion that the glyoxalase system may function in a regulatory capacity by monitoring intracellular methylglyoxal (or other α -ketoaldehydes) concentrations.¹¹

The disproportionation of α -ketoaldehydes in alkaline solution is also an intramolecular Cannizzaro reaction. This reaction has been studied extensively, especially for phenylglyoxal^{12,13,14}, and also involves migration of the aldehydic hydrogen without exchange with solvent¹³. Furthermore, Hine and Koser¹⁴ have established that the disproportionation of phenylglyoxal involves intramolecular hydride migration as the rate determining step.

A summary of their proposed reaction sequence is given in Scheme 2. Comparison

of the two reaction Schemes shows the formal similarity between the enzyme catalyzed reaction (3) and the hydroxide catalyzed reactions (7 and 8).

We have examined the effects of substituents on the hydroxide catalyzed disproportionation of a series of substituted phenylglyoxals in order to 1) test whether reactions 7 and 8 involve rate determining hydride migration for a broad series of meta or para substituents; 2) examine this reaction for Linear Free Energy Relationships between reactivity and substituent constants; 3) attempt to explain the observed reactivity by analyzing the carbonyl stretching frequencies of the aldehyde and ketone carbonyls; 4) and obtain an understanding of reactions 7 and 8 as models for the glyoxalase-I reaction (3). We recently observed that the glyoxalase-I catalyzed disproportionation of substituted phenylglyoxals is insensitive to ring substituents¹⁵. This raises the question of whether reaction 3 involves rate determining hydride migration or whether hydride migration simply shows a very small substituent effect. Reactions 7 and 8 thus become critical models for reaction 3.

Scheme 1

GSH = γ -L-glutamyl-L-cysteinylglycine

Scheme 2

Results and Discussion

The rates of disproportionation of a series of substituted phenylglyoxal hydrates, including H, *p*-CH₃, *p*-OCH₃, *p*-Br, *p*-Cl, *p*-phenyl, *m*-OCH₃ and *p*-NO₂ were measured at pH 12 by following the changes in the u.v. absorbances at the λ_{MAX} of the hydrates. Para-hydroxyphenylglyoxal was also examined. This member of the series disproportionated slowly at pH 12 and, consequently, was reacted at higher pH. The *p*-OCH₃ derivative was also disproportionated at this higher pH (ca. 0.1 M NaOH solution) and the factor $\frac{k_{\text{p-OCH}_3}}{k_{\text{p-O}^-}} = 39$ was assumed valid at pH 12. The *p*-OH derivative exists as the *p*-O⁻ anion at high pH. The pseudo first order rate constants obtained and the wavelengths employed are listed in TABLE I. There is a 3600 fold range in rate constants between the *p*-NO₂ and *p*-O⁻ derivatives, indicative of transition state stabilization by electron withdrawing groups. Figure 1 shows a Hammett plot of $\log k$ vs. σ_x^{16} for this series of compounds. A fairly good Linear Free Energy Relationship is observed. The slope, ρ , is 2.0, comparable in size and magnitude to the OH⁻ catalyzed hydrolysis of substituted methylbenzoates¹⁷. The linear relationship over this wide range of substituents suggests a common mechanism for this series of disproportionations.

In their study on the mechanism of disproportionation of phenylglyoxal hydrate, Hine and Koser¹⁴ reported that the rate determining step is the intramolecular hydride migration (reaction 7 or 8 of Scheme 2) and that reaction 8 predominates at hydroxide concentrations above 3 mM. At pH 12, both the mono- and dianion should contribute to the observed rate with the majority of reaction occurring via the dianion. The existence of a linear relationship for the entire series of substituted phenylglyoxals examined in

the present study, at pH 12, appears surprising, if both reactions 7 and 8 are involved. However, if the acidities of the hydrates (i.e., reactions 5 and 6 of Scheme 2) are insensitive to ring substituents, then the contributions of reactions 7 and 8, respectively, would be insensitive to substituents, and a linear relationship might be anticipated. To examine this question, the carbonyl stretching frequencies of the substituted phenylglyoxals were determined for the ketones in the hydrated compounds and for both the aldehydes and the ketones in the unhydrated compounds. The values are listed in TABLE II. The aldehyde carbonyl stretching frequency is $1727 \pm 2 \text{ cm}^{-1}$, totally insensitive to ring substituents, while the ketone carbonyls are quite sensitive to ring substituents, both for the hydrates and the unhydrated phenylglyoxals. If one assumes that the carbonyl stretching frequency reflects sensitivity to nucleophilic addition, one might expect that the extent of hydration of the aldehyde in aqueous solution and the pKa values of the hydrates will be similar for this entire series of phenylglyoxals. This would help explain the linear relationship observed in the rates of disproportionation at pH 12. This conclusion that the chemistry at the aldehyde group is insensitive to substituents agrees with earlier observations that the rates of addition of glutathione to the aldehyde groups of substituted phenylglyoxals (reaction 2, Scheme 1) and the dissociation constants of the resulting hemimercaptals are insensitive to ring substituents¹⁵.

If hydride migration is rate determining, and if the ketone carbonyl stretching frequencies reflect the influence of the ring substituents, linear relationships might be expected in plots of $\log k$ vs. $\nu_c = 0$. Figures 2 and 3 show plots of $\log k$ vs. the ketone carbonyl

stretching frequencies of the hydrates and the unhydrated phenylglyoxals, respectively. Fairly good Linear Free Energy Relationships are observed in both cases. The sensitivity of the reaction is about one log unit of k for a $\nu_{\text{C=O}}$ of 13 cm^{-1} . These results agree with the general conclusion that the carbonyl stretching frequency can be a good indicator of chemical reactivity. Previous studies have shown that ketone carbonyl stretching frequencies can also be good models for predicting reactivities of ester solvolyses proceeding by carbonium ion intermediates^{18,19}.

The usefulness of reactions 7 and 8 as models for the glyoxalase-I reaction (reaction 3, Scheme 1) is limited. The high sensitivity of the OH^- catalyzed disproportionation of substituted phenylglyoxals to substituents compared to the lack of sensitivity¹⁵ in the glyoxalase-I reaction suggests that hydride migration may not be the rate determining step in the enzyme reaction.

Experimental

The substituted phenylglyoxals used in this study were synthesized by the following general procedures.

Procedure A:²⁰ A substituted acetophenone as a 1-2 M solution in dioxane containing an equivalent amount of selenous acid was refluxed for 4 hours. The mixture was concentrated by rotary evaporation, and the residue was vacuum distilled. The resulting oil was added to hot water to form the crystalline substituted phenylglyoxal hydrate which was recrystallized from chloroform, acetone.

Procedure B:²¹ A slurry of a substituted phenacylbromide in acetonitrile was treated with a slight excess of AgNO_3 . The resulting mixture was stirred for 24-48 hours at room temperature, filtered, and the solvent removed by rotary evaporation. The residue (a phenacylnitrate) was dissolved in diethyl ether and washed with water. After drying over MgSO_4 , the solvent was removed, and the residue was added to dimethylsulfoxide containing about 1% sodium acetate. The mixture was stirred at room temperature for 30 minutes and then was poured into ice-water saturated with NaCl . The resulting mixture was extracted with diethyl ether, washed with water, dried over MgSO_4 and then the solvent evaporated off. The resulting substituted phenylglyoxal hydrate was recrystallized as in procedure A.

The substituted phenylglyoxal hydrates prepared by either procedure were colorless solids except for the $p\text{-NO}_2$ derivative which did not form a crystalline hydrate. The melting points, however, were observed to be somewhat variable during the recrystallization procedures. This presumably is a reflection of the extent of hydration and has also been observed by others¹⁴. All of the substituted phenylglyoxals were converted into the dioxime derivatives for elemental analysis. The data for characterization of

the series of phenylglyoxals are given in TABLE III.

Rates of Disproportionation: Phosphate buffers, pH 12, $\mu = 0.6$, were prepared using distilled, deionized water and reagent grade chemicals.

Reaction rates were monitored at the λ_{MAX} values of the substituted phenylglyoxals obtained from u.v. spectra recorded in pH 7 phosphate buffer using a Cary 15 recording spectrophotometer. In all cases, the substituted phenylglyoxals have molar extinction coefficients ca. $10^4 \text{ M}^{-1}\text{cm}^{-1}$ at the λ_{MAX} whereas the substituted mandelate products show low absorption at these wavelengths. The reaction rates were measured on a Gilford 222 recording spectrophotometer employing Beckman DU optics. The temperature was controlled with a circulating water bath. First order 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. Reactions were initiated by addition of small quantities (10-20 $\mu\text{l.}$) of 1:1 ethanol, H_2O stock solution of the substituted phenylglyoxals. These small quantities were placed on the end of a flattened stirring rod and introduced directly into the spectrophotometer cell containing 3.0 ml. of temperature equilibrated buffer. The ethanol was generally useful for preparing stock solutions of convenient concentrations. Use of stock solutions without ethanol gave the same rate data. The initial concentrations of substituted phenylglyoxals in the reaction cell were generally ca. 10^{-4} M .

Carbonyl Stretching Frequencies: The ketone and aldehyde carbonyl stretching frequencies were measured on a Perkin-Elmer 621 recording spectrophotometer using very slow scan rates and expanded scales. Generally, the range 1800-1600 cm^{-1} was scanned over a one hour period, and a polystyrene standard was added to the cell holder immediately after the carbonyl band was passed in order to accurately locate the carbonyl stretching frequency. This procedure gave

values reproducible to $\pm 1.5 \text{ cm}^{-1}$.

The ketone carbonyl stretching frequencies of the substituted phenylglyoxal hydrates were measured in Nujol mulls. The ketone and aldehyde carbonyl stretching frequencies of the unhydrated compounds were determined in dilute acetonitrile solutions. Although carbonyl frequencies are generally measured in carbon tetrachloride solutions, it was found that the unhydrated phenylglyoxals in carbon tetrachloride rapidly deteriorate, presumably by polymerization. Only a trace of water is required to initiate polymerization. Acetonitrile solutions were sufficiently stable to allow slow scanning rates to be used. The anhydrous solutions were prepared by warming acetonitrile solutions of the hydrates over molecular sieves, with repeated transfers to fresh molecular sieves.

- (1) (a) This work was supported by U.S. Public Health Service, National Cancer Institute (1R01 CA 11850-01) and U.S. Atomic Energy Commission under Sandia Corporation Contract 51-1985. An equipment grant from Research Corporation is also gratefully acknowledged. (b) A preliminary report of this work was presented at the Southwest Regional Meeting of the American Chemical Society, San Antonio, Dec., 1971. (c) Address correspondence to this author at the Department of Biochemistry, University of New Mexico School of Medicine, Albuquerque, N.M. 87106.
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TABLE I Rate Constants for the Disproportionation of Substituted Phenylglyoxals, pH 12, 25°. ^a

<u>x</u>	<u>k (10⁻⁴sec⁻¹)</u>	<u>log k</u>	<u>σ_x^c</u>	<u>λ(nm)^d</u>
H	7.60 ± 0.13	0.881	0.0	251
p-CH ₃	3.05 ± 0.05	0.484	-0.170	263
p-OCH ₃	1.37 ± 0.04	0.137	-0.268	287
p-Br	13.9 ± 0.1	1.143	+0.232	264
p-Cl	12.2 ± 0.3	1.086	+0.227	260
p-Ø	7.91 ± 0.08	0.898	-0.01	292
m-OCH ₃	10.1 ± 0.1	1.004	+0.115	255
p-NO ₂	125 ± 5	2.097	+0.778	268
p-O ⁻	0.035 ^b	-1.46	-1.00	284

a Rates measured spectrophotometrically in phosphate buffer, μ = 0.6.

b p-OH phenylglyoxal exists as the p-O⁻ derivative at high pH. Since this substituted phenylglyoxal is quite stable at pH 12, it was disproportionated at higher pH along with the p-OCH₃ compound, and the factor $\frac{p-OCH_3}{p-O^-} = 39$ was assumed applicable at pH 12.

c Values for σ_x obtained from reference (16).

d λ_{MAX} values of the substituted phenylglyoxal hydrates, pH 7. The rates of disproportionation were monitored at these wavelengths.

TABLE II. Infrared Carbonyl Stretching Frequencies of the Ketone and Aldehyde Carbonyls of Substituted Phenylglyoxals and Their Hydrates.

<u>Substituent</u>	<u>$\nu_{C=O}$ (cm⁻¹)</u>		
	<u>ketone</u>	<u>aldehyde</u>	<u>ketone(hydrated series)</u>
H	1676	1727	1699
p-CH ₃	1674	1726	1688
p-OCH ₃	1666	1728	1681
p-Br	1680	1729	1695
p-Cl	1679	1729	1695
p-Ø	1673	1726	1694
m-OCH ₃	1674	1726	1693
p-NO ₂	1688	1729	1708
p-OH	1664	1728	1681

TABLE III Characterization of Substituted Phenylglyoxals

substituent	synthetic procedure	M. P. (hydrate) °C	M. P. (dioxime)	Elemental Analysis(dioxime)		
				C	H	N
H	A	76 - 77	174 - 176	58.53 58.70	4.91 5.10	17.06 calculated 17.04 observed
p-CH ₃	A	98 - 99	166.5 - 168.2	60.67 60.81	5.66 5.63	15.96 15.87
p-OCH ₃	B	126 - 127.5	152 - 153	55.67 55.85	5.19 5.14	14.43 14.26
p-Br	B	133.5 - 134.9	167.5 - 168.5	39.53 39.74	2.90 3.14	11.52 11.39
p-Cl	B	120 - 122	159 - 160	48.38 48.28	3.55 3.73	14.10 14.33
p-Ø	B	116 - 118	216 - 218	69.99 70.02	5.03 4.94	11.66 11.59
m-OCH ₃	B	77 - 78.5	163.5 - 164.8	55.67 55.73	5.19 5.10	14.43 14.55
p-NO ₂	A	131-132/3mm ^a	186 - 188	45.94 45.92	3.37 3.54	20.09 20.40
p-OH	A	86.5-87.5	190 - 193	53.33 53.15	4.48 4.73	15.55 15.86

^aBoiling point of p-NO₂ derivative.

FIGURE 1

FIGURE LEGENDS

- FIGURE 1 Hammett plot of $\log k$, the rate constants for the disproportionation of the substituted phenylglyoxals, pH 12, vs. σ_x . Slope, ρ , is 2.0.
- FIGURE 2 Plot of $\log k$, the rate constants for the disproportionation of the substituted phenylglyoxals, pH 12, vs. the ketone carbonyl stretching frequencies of the hydrated phenylglyoxals.
- FIGURE 3 Plot of $\log k$, the rate constants for the disproportionation of the substituted phenylglyoxals, pH 12, vs. the ketone carbonyl stretching frequencies of the anhydrous phenylglyoxals.

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STERIC AND ELECTRONIC EFFECTS IN THE GLYOXALASE CATALYZED DISPROPORTIONATION OF α -KETOALDEHYDES. David L. VanderJagt* and Liang-Po B. Han* (SPON: L.F. Smith). Dept. Biochem & Chemistry, Univ. of New Mexico, Albuquerque, New Mexico 87106.

We reported previously that the glyoxalase catalyzed disproportionation of hemimercaptals of glutathione and α -ketoaldehydes is remarkably insensitive to the type of α -ketoaldehyde. A broad series of *meta* and *para* substituted phenylglyoxals (x-PG) show V_{max} values all within a factor of 4 of methylglyoxal (MG), the normal intracellular substrate. Moreover, all x-PG have smaller K_m values than MG ($3 \times 10^{-4}M$) ranging down to $2 \times 10^{-5}M$ for pCl-PG and p ϕ -PG. In all cases the dissociation constants of the hemimercaptals are similar ($10^{-3}M$). This insensitivity of V_{max} to substituents suggests that the enzyme polarizes the ketone carbonyl. With MG as substrate, there is no pH effect on V_{max} (pH 5-8) and V_{max} is identical in D₂O and H₂O, arguing against general acid catalysis as the mechanism for polarization. Ortho substituents, however, dramatically alter this insensitivity of V_{max} . Thus, 2,4-dimethyl-PG shows a V_{max} 1/2 that of pCH₃-PG and a similar K_m ($4 \times 10^{-5}M$). However, 2,4,6-trimethyl-PG is essentially inactive as a substrate. It inhibits the glyoxalase reaction but with a rather large K_i suggesting that the subtle steric effect of the second ortho substituent affects both binding and catalysis. (Supported in part by USPHS, NIH Grant 1 R01 CA11850-01, and AEC under Sandia Contract 51-1985.)

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INHIBITION OF THE GROWTH OF E. COLI BY α -KETOALDEHYDES

The ability of methylglyoxal to act as a general growth inhibition of both bacteria and mammalian cells is well established. In general, if the concentration of methylglyoxal is not too high, the inhibition is non-lethal. Several factors have been reported concerning this inhibition:

1. It is readily reversible by addition of equimolar amounts of cysteine.
2. The growth pattern of cells, especially bacteria, is interrupted only temporarily when methylglyoxal is added. After a period of time, normal growth resumes.

The present study was concerned with answering the following questions:

1. Is the inhibition of growth of *E. coli* by α -ketoaldehydes related to the nature of the α -ketoaldehyde. In particular, is there a relationship between the ability of an α -ketoaldehyde to inhibit cell growth and its ability to be a substrate in the glyoxalase system?
2. After the period of inhibition of growth is finished and the cells resume normal growth, do the cells contain a high constitutive level of same enzyme(s) involved in the reversal of the inhibition or do mutations occur?

RESULTS

Several substituted phenylglyoxals which are substrates for the glyoxalase system were examined as inhibitors of E. coli. No large variations in their inhibitor potentials were observed. All of the compounds tested are as good as or better than methylglyoxal (mole for mole) as inhibitors. These compounds also show no large variations in their substrate abilities in the glyoxalase system. However, 2,4,6-trimethylphenylglyoxal also inhibits E. coli as effectively as the other α -ketoaldehydes but is not a glyoxalase substrate. Consequently, the period of inhibition is not directly related to the time required for glyoxalase to disproportionate the α -ketoaldehyde inhibitor.

Using phenylglyoxal as a representative aromatic α -ketoaldehyde, the effects of repeated challenges to the cells by inhibitor were tested for several generations. No significant changes in sensitivity to inhibitor were found for 5 generations, suggesting that the cells do not build up high constitutive levels of whatever enzyme(s) are involved in removing the inhibitor and suggesting that the cells do not mutate. Representative graphs are shown in Fig. 1-3.

FIGURE LEGEND

Various generations of growth curves of E. coli K12 are shown in absence or presence of 1mM phenylglyoxal. The general procedure is to grow E. coli in a minimal medium and to make the solution 1mM in inhibitor as soon as the curve enters log phase. This curve is then compared to a curve where no inhibitor is added. When the top of the log phase is reached, this is referred to as a one generation of growth curve. The E. coli are then diluted in fresh minimal medium and the curves followed again.

FIGURE 1

CURVE A - 2nd generation growth curve without inhibitor in either generation.

CURVE B - 2nd generation growth curve where inhibitor was added to the 1st generation but not to the 2nd.

CURVE C - 2nd generation growth curve where both generations were inhibited.

CURVE D - 2nd generation growth curve where inhibitor was not added to the 1st generation but was added to the 2nd generation curve.

FIGURE 2

CURVE A - 3rd generation growth curve without inhibitor in any of the curves.

CURVE B - 3rd generation growth curve where inhibitor was added only in the 3rd generation.

CURVE C - 3rd generation growth curve where inhibitor was added to each generation curve.

FIGURE 3

Repeat of FIGURE 2 in the 5th generation, i.e. CURVE A never felt inhibitor, CURVE B received inhibitor only in the 5th generation and CURVE C received inhibitor in every generation curve.

FIGURE 1

FIGURE 2

FIGURE 3