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

David L. VanderJagt†

Liang-Po B. Han

Charles H. Lehman

DISCLAIMER

* From the Departments of Biochemistry and Chemistry, University of New Mexico, Albuquerque, N.M. 87106. This work was supported by U.S. Public Health Service, National Institutes of Health (Grant 1 RO1 CA11850-01) and U.S. Atomic Energy Commission under Sandia Corporation Contract 51-1985. An equipment grant from Research Corporation is gratefully acknowledged. A preliminary report of this work was presented at the Southwest Regional Meeting of the American Chemical Society, San Antonio, December, 1971.

† Author to whom correspondence should be addressed, Department of Biochemistry, University of New Mexico School of Medicine, Albuquerque, N.M. 87106.

[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 (MG), phenylglyoxal (PG), and a series of meta or para substituted phenylglyoxals (xPG) including p-CH_3, p-OCH_3, m-OCH_3, p-Br, p-Cl, p-OH, p-NO_2, and p-phenyl. For MG, $K_{diss} = 3.0 \pm 0.5 \times 10^{-3}$ M in the pH range 5-9; for PG, $K_{diss} = 0.60 \pm 0.05 \times 10^{-3}$ M, increasing somewhat at the higher end of this pH range. At pH 7, all the xPG show K_{diss} ca. 1-3 x 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-hydroxycarboxylic 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 MG-GSH adduct, again showing complete insensitivity to ring substituents. However, the K_M values for the xPG-GSH adducts (2×10^{-4} to 2×10^{-5} M) are smaller than K_M for the MG-GSH adduct

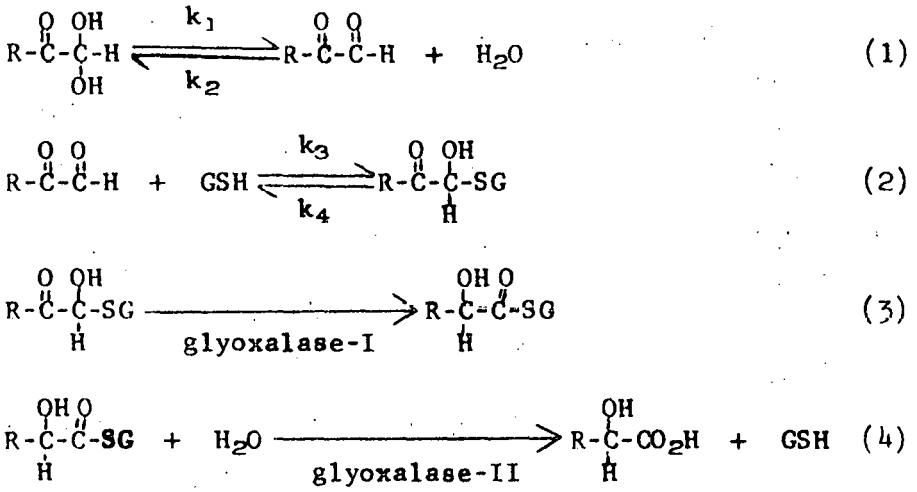
(3×10^{-4} M). The glyoxalase-I reaction is inhibited competitively by free GSH, Ki = 5×10^{-3} M. Although the glyoxalase-I reaction is known to involve intramolecular hydride transfer, the marked insensitivity of the reaction to variations in the α-ketoaldehydes suggests that hydride transfer may not 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; Neuberg, 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 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-lactoylglutathione 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 hemimercaptal 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 diluted 1:1 with water and vacuum distilled. The fraction boiling 30-34°/21 mm Hg was collected, dissolved in water, and passed through an Amberlite CG-400 ion exchange column (bicarbonate form) to remove any lactic acid present. 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) 4.14×10^4 $M^{-1}cm^{-1}$. Another method involved preparation of the bis-semicarbazone derivative which at pH 7 showed λ_{MAX} 283 nm, ϵ 2.55×10^4 (lit. λ_{MAX} 286, ϵ 3.20×10^4; Alexander and Boyer, 1971); the two methods gave good agreement. Phenylglyoxal hydrate (Aldrich) was recrystallized from chloroform, m.p. 76-77°. A series of meta or para substituted phenylglyoxals including p-CH_3, p-OCH_3, p-Br, p-Cl, p-phenyl, m-OCH_3, and p-NO_2 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, K_{diss}, of the adducts of GSH and α-ketoaldehyde are defined as

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

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. Thus,

$$Abs = [C_\alpha - x]\epsilon_\alpha + [C_{GSH} - x]\,\epsilon_{GSH} + [x]\epsilon_{AD}$$

where C_α and C_{GSH} are the initial concentrations of α-ketoaldehyde and GSH, respectively, x is the equilibrium concentration of adduct, and ϵ_α, ϵ_{GSH} and ϵ_{AD} are the respective molar extinction coefficients at wavelength λ. Then the dissociation constant can be defined as

$$K_{diss} = [C_{\alpha} - x]\,[C_{GSH} - x]/[x]$$

For the reaction between methylglyoxal and GSH, K_{diss} was determined at 240 nm, 25°, by measuring the change in absorbance when a known concentration of methylglyoxal was added to a solution of known concentration of GSH with the same concentration of GSH in the reference cell of a double beam spectrophotometer (Cary 15). The concentration of adduct can be related to the difference in absorbance between the cells if the ϵ values are known. For methylglyoxal, ϵ_{MG} 4 $M^{-1}cm^{-1}$ through the pH range 5-9 (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 [GSH]/[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). Confidence in this value of ϵ_{AD} came from the determination that >95% of possible adduct should have been present when ϵ_{AD} was determined, using the K_{diss} values obtained. Furthermore the results for a series of determinations of K_{diss} using different initial concentrations of GSH and methylglyoxal gave a consistent value for K_{diss} only if $\epsilon_{AD} = 440 \pm$ ca. 10%. 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 substituted phenylglyoxals, K_{diss} values could be determined at wavelengths where GSH does not absorb. Again, ϵ_{AD} was estimated by pushing adduct formation toward completion. 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 II. U.v. spectra were obtained using a Cary 15 recording spectrophotometer. Absorbance measurements for determinations of K_{diss} were obtained either with the Cary 15 or with a Gilford 222 modified Beckmann DU. Both spectrophotometers were temperature controlled with circulating water baths.

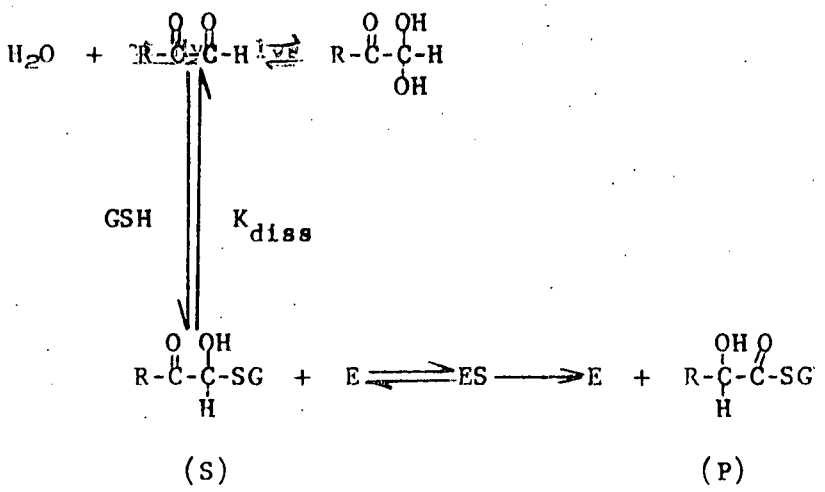
Reaction Kinetics for Adduct Formation: Reaction rates were monitored with the spectrophotometers mentioned above. All rate constants were from computer calculated least squares lines of first order plots; correlation coefficients were generally better than 0.999. Buffers were prepared from commercially available reagents and distilled, deionized water. Measurements of pH were made on a Sargent Model DR pH meter with a glass electrode.

Enzyme Kinetics: 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 $M^{-1}cm^{-1}$ (lit. ϵ 3370, Racker, 1951; ϵ 3300, Cliffe and Waley, 1961). Initial rates were obtained from the initial changes in absorbance as $\frac{dP}{dt} = \frac{\Delta \text{ Abs.}}{\Delta t(\epsilon_p - \epsilon_{AD})}$. The contribution to the starting absorbance from methylglyoxal is negligible. (ϵ_{MG} 4 $M^{-1}cm^{-1}$) and the contribution from GSH was minimized by setting up the pre-enzymic equilibrium using larger initial concentrations of methylglyoxal than of GSH. 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 μmoles/min/mg., was generally diluted 250 fold in pH 7.0 phosphate buffer containing 100 μg/ml. bovine serum albumin. This glyoxalase solution was used as a stock solution and was sufficiently stable so that after initial standardization, the solution could be used for 24

hours with less than 3% change in activity. In all initial rate studies and in the standardization of the glyoxalase stock solution, 20 µl. stock/3 ml. 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 disproportion of the GSH adducts of substituted phenylglyoxals were followed at the apparent isosbestic points of the substituted phenylglyoxal hydrates and their GSH adducts. Stock solutions (10^{-2}M) of substituted phenylglyoxals were prepared in 1:1 ethanol-water. Generally, 5-100 µl. stock were used per 3 ml. reaction volume in the initial rate studies. The effects of the ethanol were tested and found to be negligible. 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 (λiso) than do the reactants. The reaction scheme for kinetic analysis of the substituted phenylglyoxals is as follows:



Initial rates, $\frac{dP}{dt} = \frac{\Delta Abs_{\lambda iso}}{\Delta t(\epsilon_{iso} - \epsilon_p)}$ where $\Delta Abs_{\lambda iso}$ is the initial change in absorbance at the isosbestic point in time Δt, and $(\epsilon_{iso} - \epsilon_p)$ is the difference in molar extinction coefficients of reactant (S) and thiolester product (P). Knowledge of K_{diss} allows one to calculate [S] for each initial rate measured. Computer programs were written to calculate [S] (i.e. adduct) for various initial concentrations of GSH and substituted phenylglyoxal and to convert $\frac{\Delta Abs}{\Delta t}$ into rates in Mt^{-1}. Owing to the rapidity of the pre-enzymic reactions (1 and 2 of Scheme 1), use of any wavelength other than λiso 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 λiso and ϵ values are given in TABLE III.

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 IV). For the methylglyoxal adduct, $K_{diss} = 3.0 \pm 0.5 \times 10^{-3}$ M throughout the pH range studied. The adduct of phenylglyoxal shows $K_{diss} = 0.60 \pm 0.05 \times 10^{-3}$ 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 V. 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 VI 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 acetonitrite were warmed over molecular seives to prepare the unhydrated α-ketoaldehydes. Infrared spectra showed that the carbonyl stretching frequency for the aldehyde carbonyl was essentially constant at $1727 \pm 2\ cm^{-1}$, totally insensitive to substituent; the ketone carbonyl stretching frequencies were very sensitive to substituents[1]. 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. FIGURE 3 shows a comparison of two typical runs where in one case the pre-enzymic reactions were set up so that at each point the amount of adduct at equilibrium exceeded the amount of free GSH, while in the other run, no preference was given to the initial GSH/methylglyoxal ratio. The presence of free GSH inhibits the glyoxalase reaction (Kermack and Matheson, 1957). Determination of the enzyme-inhibitor dissociation constant, Ki, and type of inhibition by direct evaluation of the double reciprocal plots (FIGURE 3) is difficult. Ki was determined by working under saturation

conditions. Initially a high methylglyoxal/GSH ratio was used to determine V_{MAX} under conditions of negligible free GSH. Then increasing amounts of GSH were added and the apparent V_{MAX} observed. At that point where the apparent V_{MAX} is one-half of the initial V_{MAX}, $Ki = K_M \frac{[GSH]}{[adduct]}$. The value of $[GSH]/[adduct]$ at that point is about 16. For the methylglyoxal adduct, $K_M = 3 \times 10^{-4}$ M, obtained from double reciprocal plots in runs using limited amounts of GSH (FIGURE 3). The value Ki for GSH inhibition is therefore about 5×10^{-3} M, and the inhibition is competitive.

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 VII. The K_M values are smaller than K_M for the methylglyoxal adduct, ranging down to 2×10^{-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}). In the simplified Michaelis-Menten (1913) scheme (eq. 5)

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (5)$$

$K_M = \frac{k_2 + k_3}{k_1}$; $V_{MAX} = k_3[E_{total}]$. The insensitivity of V_{MAX} to variation in α-ketoaldehyde implies similar catalytic rate constants, k_3, for all of these substrates. Therefore the K_M values will parallel the actual ES dissociation constants (k_2/k_1) indicating that the substituted phenylglyoxal adducts bind to the enzyme better than does the methylglyoxal adduct. A representative double reciprocal plot, using phenylglyoxal as substrate, is shown in FIGURE 4. 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.

In addition to the spectral changes which occur during the disproportionation reactions, and the observed broad tailing absorption around 240 mu (FIGURE 2) characteristic of thiol esters (Racker, 1951), products were checked by hydrolysis of the thiol esters at pH 10.5. TABLE VIII shows the pseudo first order rate constants for hydrolysis of the GSH esters of methylglyoxal and the substituted phenylglyoxals. The rates are quite similar as

expected for these compounds because the variations in the side groups are quite far removed from the ester linkages.

Discussion

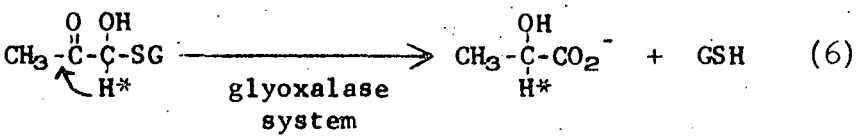
The glyoxalase system consists of two enzymes, glyoxalase-I (S-lactoyl-glutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5) and glyoxalase-II (S-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6). The present study on the substrate requirements for glyoxalase-I supports the conclusion of Davis and 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-

hydic hydrogen (eq. 6), presumably by



(6)

intramolecular hydride migration. If intramolecular hydride migration is rate determining and sensitive to the polarity of the α-ketone carbonyl, one might expect to observe some substituent effects. The lack of sensitivity to substituents observed in our study of substituted phenylglyoxals as substrates may mean that hydride migration is not the rate determining step. However, it is possible the hydride migration is facilitated through polarization of the α-ketone group by the enzyme in which case the added effects on polarization by the substituents may be small, and hydride migration could still be rate determining.

The smaller K_M values for the substituted phenylglyoxal adducts generally parallel the size and hydrophobic nature of the substituents, K_M being smallest for the p-phenyl and p-chloro substituents. This observation is in accord with a recent report (Vince and Wadd, 1969) of a non-polar region at or near the active site of glyoxalase-I, based upon the observed ability of S-alkylglutathiones to increasingly inhibit glyoxalase-I as the size of the alkyl chain increased.

The question of the biological role of glyoxalase remains

open. The broad specificity of the enzyme for many α-keto-aldehydes 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.

Acknowledgment: The authors thank the University of New Mexico Computing Center for generous donation of computer time.

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

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

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

Dakin, H.D., and Dudley, H.W. (1913a), J. Biol. Chem. 14, 155.

Dakin, H.D., and Dudley, H.W. (1913b), J. Biol. Chem. 14, 423.

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

Egyud, L.G., and Szent-Gyorgyi, A. (1966a), Proc. Natl. Acad. Sci. U.S. 55, 388.

Egyud, L.G., and Szent-Gyorgyi, A. (1966b), Proc. Natl. Acad. Sci. U.S. 56, 203.

Franzen, V. (1956), Chem. Ber. 89, 1020.

Hopkins, F.G., and Morgan, E.J. (1948), Biochem. J. 42, 23.

Kallen, R.G., and Jencks, W.P. (1966), J. Biol. Chem. 241, 5851.

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

Kornblum, N., and Frazier, H.W. (1966), J. Am. Chem. Soc. 88, 865.

Knox, W.E. (1960), in The Enzymes, Boyer, P.D., Lardy, H., and Myrback, K., Eds., New York, Academic Press, Vol. 2, p. 253.

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

Lineweaver, H., and Burk, D. (1934), J. Am. Chem. Soc. 56, 658.

Lohmann, K. (1932), Biochem. Z. 254, 332.

Michaelis, L., and Menten, M. (1913), Biochem. J. 49, 333.

Neuberg, C. (1913), Biochem. Z. 49, 502.

Platt, M.E., and Schroeder, E.F. (1934), J. Biol. Chem. 104, 281.

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

Racker, E. (1952), Biochim. Biophys. Acta 9, 577.

Riley, H.A., and Gray, A.R. (1943), Org. Syn., Col. Vol. II, 509.

Rose, I.A. (1957), Biochim. Biophys. Acta 25, 214.

Salem, H.M. (1954), Biochem. J. 57, 227.

Sparkes, B.G., and Kenny, C.P. (1969), Proc. Natl. Acad. Sci. U.S. 64, 920.

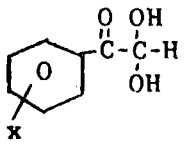
Szent-Gyorgyi, A., Egyud, L.G., and McLaughlin, J.A. (1967), Science 155, 539.

Szent-Gyorgyi, A. (1968), Science 161, 988.

Vince, R., and Wadd, W.B. (1969), Biochem. Biophys. Res. Commun. 35, 593.

TABLE I

U.V. Data for Substituted Phenylglyoxal Hydrates[a]



substituent (x)	λMAX (nm)	log ε
H	251	4.01
p-CH_3	263	4.15
p-OCH_3	287	4.18
p-Br	264	4.17
p-Cl	260	4.15
p-phenyl	292	4.26
m-OCH_3	255	3.89
p-NO_2	268	4.11
p-OH	284	4.10

[a] Spectra recorded at 25 °C, phosphate buffer, pH 7.0, μ = 0.2

TABLE II

U.V. Data for Determination of the Dissociation Constants, K_{diss}, of the Adducts of Glutathione and Substituted Phenylglyoxals and Methylglyoxal[a]

substituent	λ (nm)	ϵ_{AD} $(M^{-1}cm^{-1})$[b]	ϵ_{xPG}[c]
H	280	2450	1280
p-CH_3	320	1250	165
p-OCH_3	320	4190	1370
p-Br	310	1955	280
p-Cl	320	1350	380
p-phenyl	340	3180	795
m-OCH_3	310	2060	2550
p-NO_2	340	1190	575
p-OH	320	5735	4490
methylglyoxal	240	440	4

[a] Spectra recorded at 25 °C, phosphate buffer, pH 7.0, μ = 0.2;

[b] ϵ_{AD} is the molar extinction coefficient estimated for the adduct at wavelength λ using high α-ketoaldehyde/GSH;

[c] ϵ_{xPG} is the molar extinction coefficient measured for the substituted phenylglyoxal at wavelength λ.

TABLE III

Apparent Isosbestic Points for the Substituted Phenylglyoxal Hydrates and their GSH Adducts[a]

substituent	λ (nm)	ϵ_{iso}[b]	ϵ_p[c]
H	263	6,790	1,100
p-CH_3	273	10,300	1,010
p-OCH_3	296	12,380	970
p Br	272	11,500	1,860
p-Cl[d]	270	10,000	1,020
p-phenyl	303	16,000	2,280
m-OCH_3	263	5,230	1,670
p-NO_2	270	13,000	10,700
p-OH	297	9,930	1,140

[a] Spectra recorded at 25 °C, phosphate buffer, pH 7.0, μ = 0.2;

[b] ϵ_{iso} is the molar extinction coefficient ($M^{-1}cm^{-1}$) at the isosbestic wavelength λ;

[c] ϵ_p is the molar extinction coefficient of the thiol ester product formed in the glyoxalase-I reaction at the isosbestic wavelength;

[d] the p-Cl derivative did not exhibit an isosbestic point, although the hydrate and adduct are almost at an isosbestic point at 270 nm.

TABLE IV

Dissociation Constants of the GSH Adducts of Methylglyoxal and Phenylglyoxal as a Function of pH[a]

pH	buffer	[MG] mM	[GSH] mM	no. det.	K_{diss} (mM)
5.25	suc.	3.32-8.03	1.67-3.27	7	2.8±0.4
5.28	acet.	3.32-8.03	1.63-3.19	5	3.3±0.3
5.75	suc.	3.32-8.03	1.86-3.68	6	3.1±0.3
6.18	phos.	3.32-8.03	1.62-3.20	7	2.6±0.2
6.85	phos.	3.24-10.0	0.52-3.24	8	2.5±0.4
7.47	phos.	3.32-8.03	1.53-3.03	5	3.5±0.6
8.26	phos.	3.32-8.03	1.49-2.91	4	3.0±0.2
8.98	carb.	3.32-6.64	0.41-1.22	5	3.5±0.2
pH	buffer	[PG] mM	[GSH] mM	no. det.	K_{diss} (mM)
5.25	suc.	0.448-0.882	0.68-1.58	6	0.58±0.06
5.28	acet.	0.830-1.60	0.64-1.58	5	0.48±0.05
5.74	suc.	0.448-0.880	0.74-3.50	4	0.55±0.04
6.18	phos.	0.448-0.868	0.64-3.16	8	0.55±0.02
6.85	phos.	0.580-1.35	0.65-3.25	7	0.57±0.07
7.47	phos.	0.455-0.882	0.65-1.63	5	0.64±0.05
8.26	phos.	0.830-1.60	0.56-1.43	5	0.72±0.04
8.98	carb.	0.830-1.60	1.57-2.31	5	0.91±0.06

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

TABLE V

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

substituent	[xPG] x 10^{-4} M	[GSH] mM	no. det.	K_{diss} (mM)
p-CH_3	7.24-7.56	0.44-5.97	9	1.1±0.1
p-OCH_3	0.70-0.72	0.46-2.89	5	1.4±0.1
p-Br	0.52-2.60	0.25-1.97	5	1.5±0.2
p-Cl	1.43-3.92	0.74-2.22	5	3.6±0.5
p-phenyl	0.18-0.30	0.22-1.09	4	0.89±0.05
m-OCH_3	0.72-1.80	0.25-2.00	5	1.2±0.1
p-NO_2	1.73-3.46	0.55-1.36	4	1.2±0.2
p-OH	0.34-0.85	0.51-3.90	4	0.93±0.05

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

TABLE VI

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

substituted phenylglyoxal[b]	k (10^{-3} sec^{-1})
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 in added KCl);

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

TABLE VII

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

α-ketoaldehyde	K_M (M)	V_{MAX} (relative)[a]
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 in added KCl)

TABLE VIII

Rate Constants for Hydrolysis of the Thiol Ester Products of the Glyoxalase-I Disproportionation of Methylglyoxal and the Substituted Phenylglyoxals, pH 10.5[a]

substituted phenylglyoxal[b]	k (10^{-3} sec^{-1})
H	1.6
p-CH_3	3.3
p-OCH_3	3.2
p-Br	5.5
p-Cl	5.9
p-phenyl	3.7
m-OCH_3	3.6
p-OH	0.9
methylglyoxal	6.8

a All reactions run on a pH-stat, under nitrogen, at 25°.

b p-NO_2 derivative represents a special case and is not included. However, its u.v. spectrum was consistent with thiol ester formation.

FIGURE LEGENDS

FIGURE 1. Spectrum of a 10^{-4} M solution of phenylglyoxal (Curve A), pH 7, 25 °C, and spectra of equilbrium 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 3a. Representative double reciprocal plot for the glyoxalase-I catalyzed disproportionation of the GSH adduct of methylglyoxal under conditions where the concentrations of adduct at each point exceed the concentrations of free GSH; pH 7, 25°.

FIGURE 3b. Double reciprocal plot for the methylglyoxal reaction as in FIGURE 3a but with no consideration given to the concentrations of free GSH in equilbrium with the adduct.

FIGURE 4. Representative double reciprocal plot for the glyoxalase-I catalyzed disproportionation of the GSH adduct of phenylglyoxal, monitored at the apparent isosbestic point; pH 7, 25°.

FIGURE 1

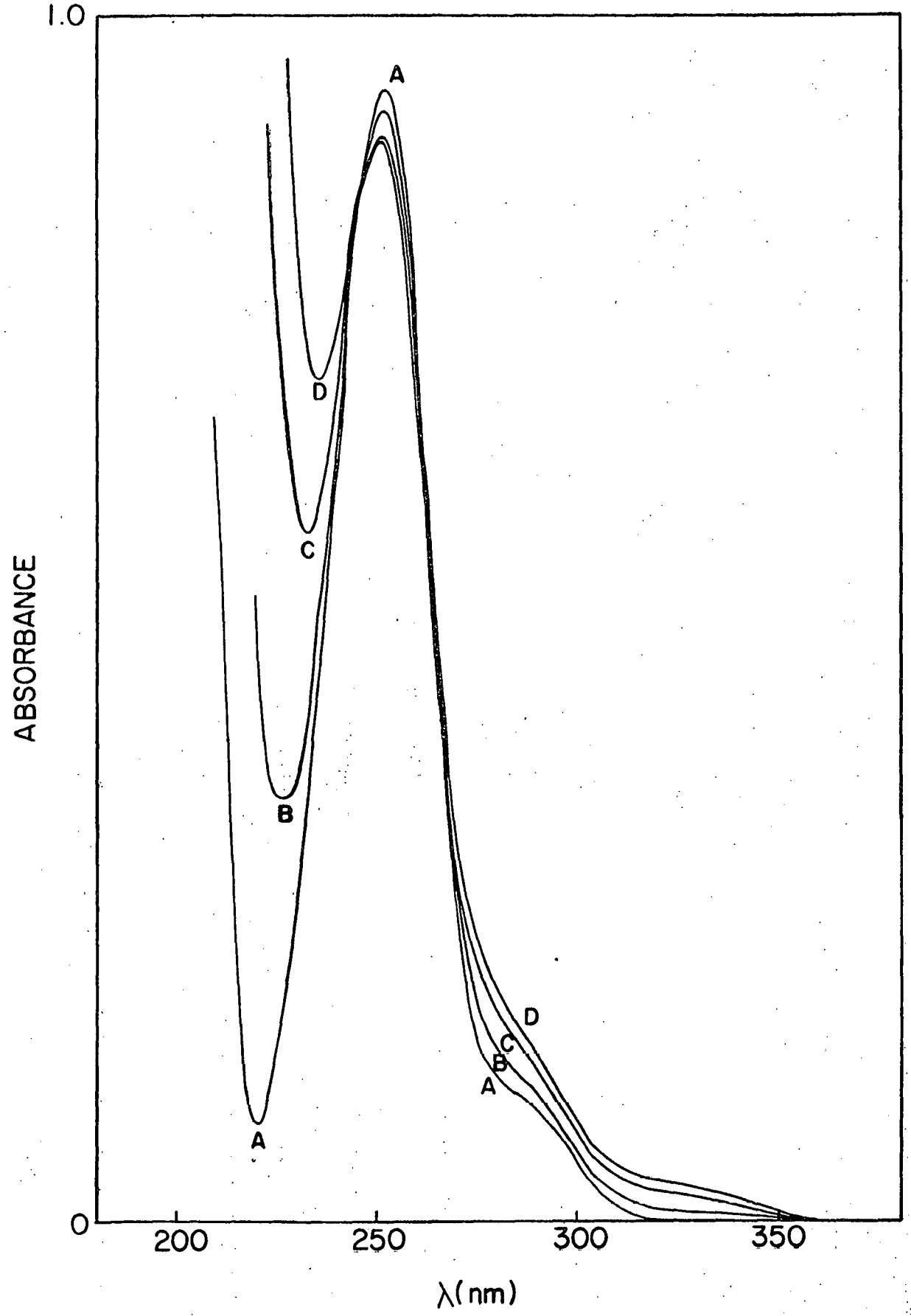


FIGURE 2

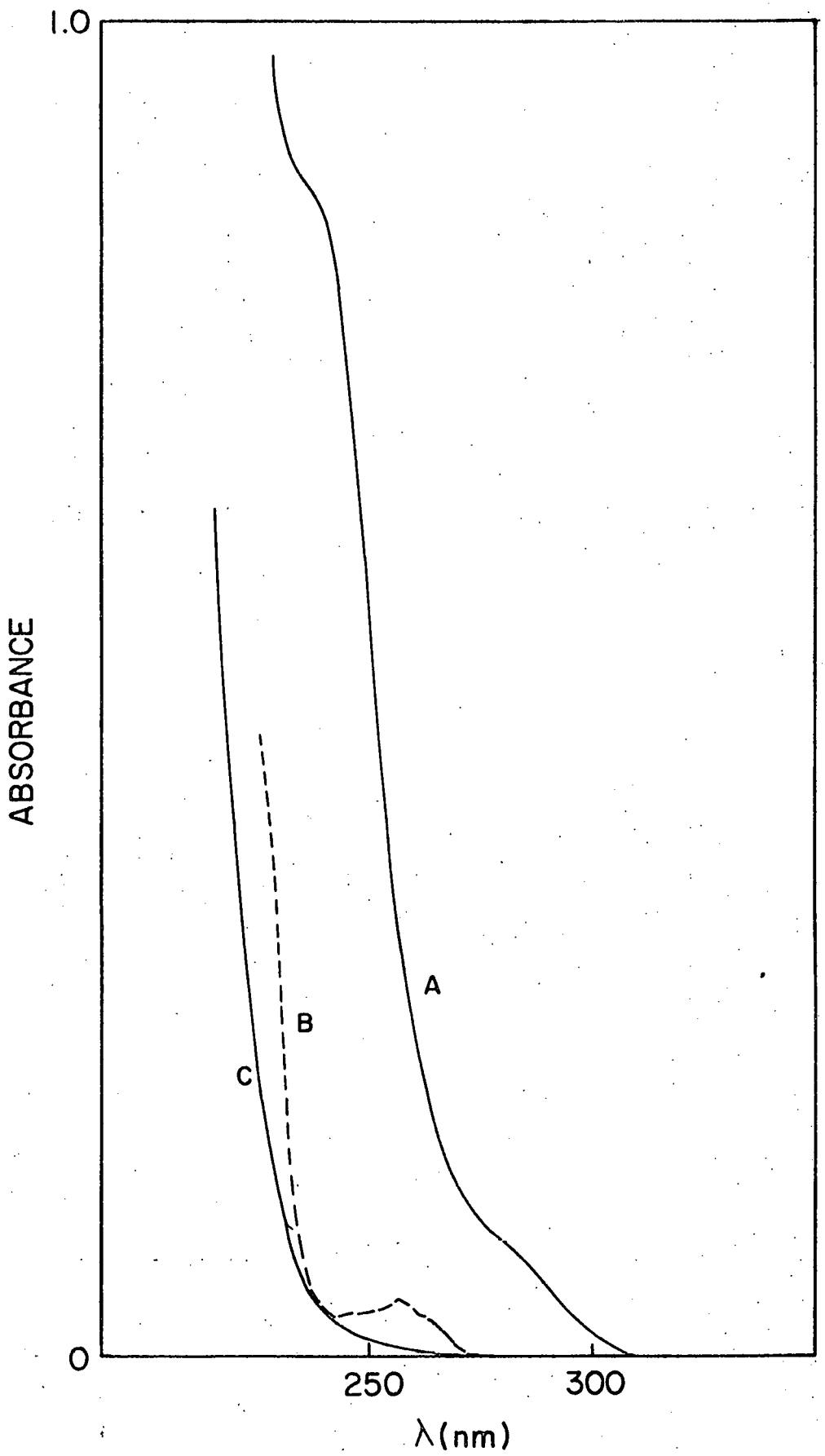


FIGURE 3a

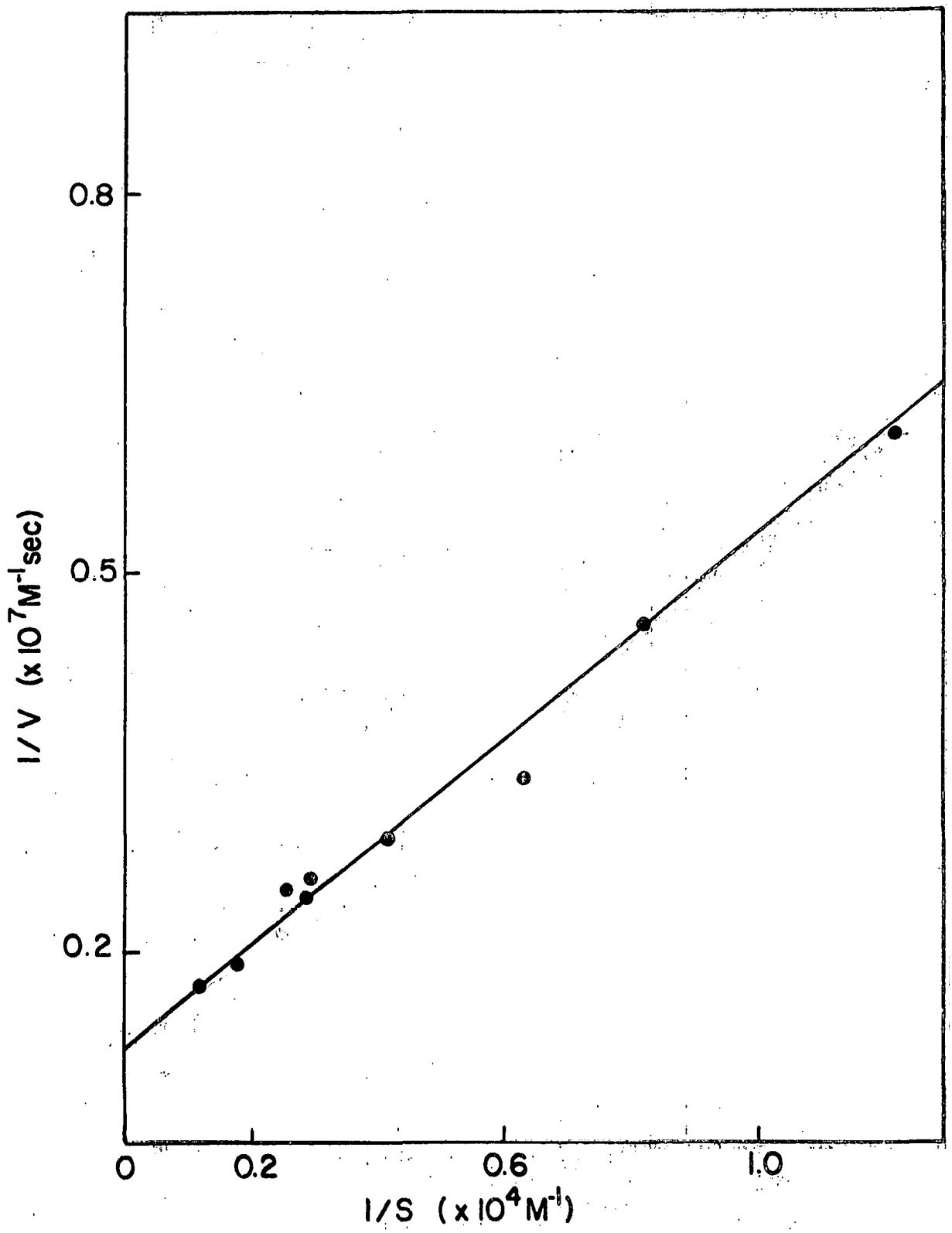


FIGURE 3b

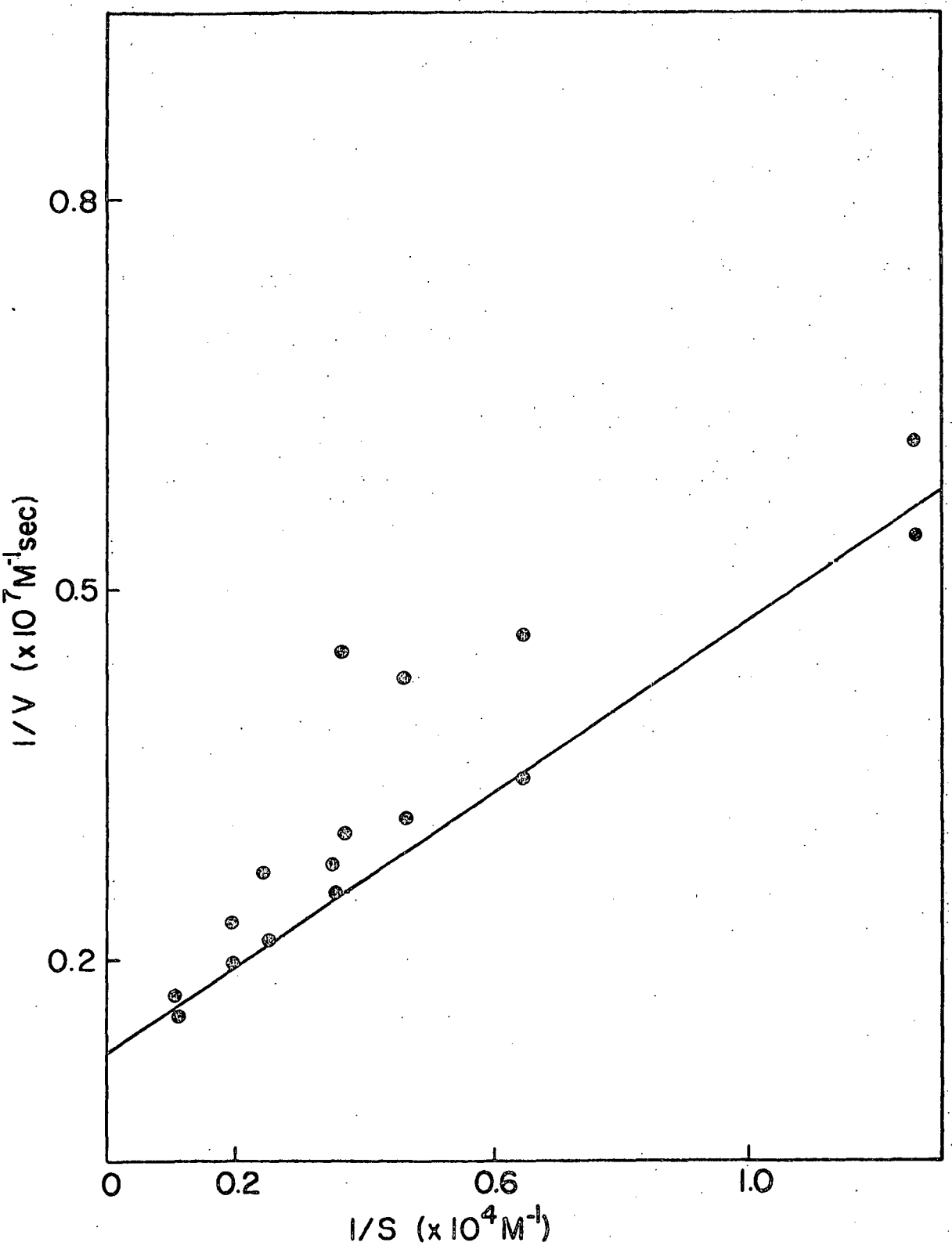


FIGURE 4