Hammett Analysis of the Human Erythrocyte Glyoxalase I-Catalysed Rearrangement to Mandeloyl Thiolesters of Hemithioacetals formed from Glutathione and Substituted Arylglyoxals†

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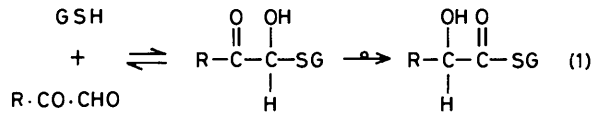
Glyoxalase I (EC 4.4.1.5), purified to homogeneity from human erythrocytes, catalyses the rearrangement to α-hydroxymandeloylglutathiones of the hemithioacetals formed by equilibration of glutathione with *para*-substituted arylglyoxals. The association constants describing these equilibria were measured at 25 °C, pH 7.0. Values of k_{cat}, K_m, and k_{cat}/K_m, describing the substrate behaviour of these hemithioacetals towards human erythrocyte glyoxalase I, were determined at pH 7.0 and 25 °C. Hammett analysis showed a linear correlation only for k_{cat} (ρ +0.43) *versus* Hammett σ constants. Literature data for glyoxalase I from yeast showed similar behaviour (ρ +0.47). In both cases good correlation required exclusion of the unsubstituted phenylglyoxal data and consideration only of *para*-substituted substrates. A linear free energy relationship was found between k_{cat} values for the human erythrocyte glyoxalase I-catalysed reaction and the rearrangement step for the model, non-enzymatic reaction. Comparisons of model and enzymatic cases on the basis of Hammett sensitivities and primary deuterium isotope effects indicated that both model and enzymatic transition states are probably closely similar and involve rate-determining deprotonation of the C–H bond adjacent to the sulphur atom in the hemithioacetal.

Glyoxalase I (EC 4.4.1.5) catalyses the rearrangement of a glutathione–α-ketoaldehyde-derived hemithioacetal [equation (1)] to the corresponding α-hydroxythiolester, a reaction of



interest for a number of reasons.[1] First, the rearrangement is an α-ketol type and probably occurs *via* an enediolate ion intermediate.[1] Secondly, the substrate α-ketoaldehydes are indigenously cytotoxic in some cases, and there are a number of groups designing cytotoxins and antitumour drugs based on the glyoxalase I reaction.[1,2] Several classes of glyoxalase I inhibitor have been reported to be active in antitumour tests at various levels. This has led to an interest in mechanism-based inhibitors of this system and the transition-state (or partial transition-state) analogue approach has been quite successful in this field.[3]

A prerequisite for such approaches to enzyme inhibition is a detailed knowledge of the mechanism of action of the target enzyme. This has been reviewed[1] for glyoxalase I, which appears to function by generating a carbanion (an enediolate ion) from the hemithioacetal by proton abstraction from the carbon atom proximal to sulphur [here C(1)], mediated by an active-site base, as yet unidentified. Reprotonation at C(2), distal to the sulphur, leads to the product.

Detailed physical organic studies have been reported for a model of reaction (1), the diazabicyclo[2.2.2]octane-catalysed rearrangement of hemithioacetals.[4–6] These studies, incorporating Hammett and related analyses as well as kinetic solvent deuterium and primary isotope effects, led to a similar mechanistic (enediolate ion) view of the model reaction as described above for the enzyme case. We now report the first, detailed Hammett-type analysis of the human erythrocyte glyoxalase I-catalysed rearrangement of the glutathione hemithioacetals of a series of substituted arylglyoxals XC_6H_4COCHO.

Experimental

Materials and Methods.—Glyoxalase I, purified from human erythrocytes following the procedure of Aronsson *et al.*,[7] had a specific activity at 25 °C at pH 6.60 of 858 μmol of substrate converted per minute per mg of protein. The enzyme was homogeneous (single band) by the criteria of anodic discontinuous polyacrylamide and sodium dodecyl sulphate–polyacrylamide (10—18% gradient) electrophoresis. The enzyme activity was assayed using methylglyoxal and glutathione as substrates as previously described.[3,7] Methylglyoxal was purified before use and assayed as described;[3] glutathione (from Sigma Chemical Co., St. Louis) was used as received and its thiol titre determined by Ellman titration.[8]

Substituted arylglyoxals were synthesised, as previously described,[6] by selenium(IV) oxide oxidation of the correspondingly substituted acetophenone, following the method of Riley and Gray.[9] Products were characterised, as described,[6] and had satisfactory n.m.r. (^1H, ^{13}C) and i.r. spectra, and were pure by h.p.l.c. Dimethyl sulphoxide was refluxed and distilled from anhydrous calcium hydride and stored before use over molecular sieves under nitrogen in the dark. All buffers were prepared using glass-distilled deionised water and using AnalaR, or equivalent quality, reagents. Details of rate measurements are given in the results section. Data were analysed by the Enzfitter© program of R. J. Leatherbarrow, distributed by Elsevier Biosoft.

Results

Measurement of Association Constants for GSH with Arylglyoxals.—Glyoxalase I processes the hemithioacetal component of the equilibrium between GSH and arylglyoxals [equation

† Abbreviations used: GSH, reduced glutathione; DMSO, dimethyl sulphoxide; DABCO, 1,4-diazabicyclo[2.2.2]octane; MOPS, 3-(*N*-morpholino)propanesulphonic acid.

Table 1. U.v. spectral data for substituted arylglyoxal hydrates, their adducts with GSH, and the thiolester products of the glyoxalase I-catalysed reaction[a]

Substituent	$\lambda_{max.}$	ε[c]	λ_{eq}[d]	λ_{iso}	ε_s[e]	ε_p[e]
H	251	10 300	280	263	6 790	1 100
4-CH_3	263	14 200	320	273	10 300	1 010
4-OCH_3	287	15 200	320	296	12 380	970
4-Br	264	14 800	310	272	11 500	1 860
4-Cl	260	14 100	320	270	10 000	1 020
4-C_6H_5	292	18 000	340	303	16 000	2 280
4-NO_2	268	13 000	340	270	13 000	10 700
4-CF_3[b]	282	13 600	320	291	10 800	1 230

[a] Values from ref. 10. Spectra recorded at 25 °C, phosphate buffer, pH 7.0, μ 0.2, half of ionic strength from buffer and half from added KCl. [b] Values obtained by identical method to that used ref. 10. [c] ε (M^{-1} cm^{-1}) is the molar extinction coefficient at $\lambda_{max.}$ (nm), the wavelength of maximum absorbance used to determine arylglyoxal concentrations. [d] λ_{eq} (nm) is the wavelength used to determine the association constants (K_{ass}) for GSH and substituted-arylglyoxals. [e] ε_s (l mol^{-1} cm^{-1}) is the molar extinction coefficient of the hemithioacetal substrate and ε_p (l mol^{-1} cm^{-1}) is the molar extinction coefficient of the thiolester product, both at the isosbestic wavelength, λ_{iso} (nm).

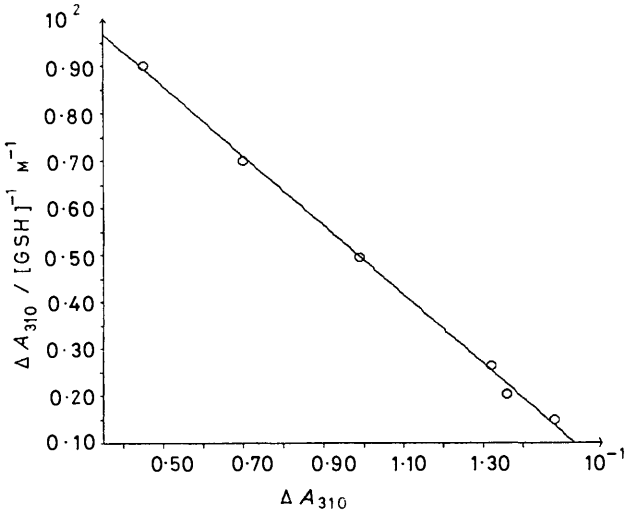


Figure 1. Scatchard plot of ΔA_{310}/[GSH] *versus* ΔA_{310} for determination of K_{ass} for the equilibrium between GSH and *p*-bromophenylglyoxal. Points are experimental; line is by least-squares regression analysis (see text for details)

(1)] and, as determination of Michaelis–Menten parameters requires accurate knowledge of substrate concentrations, a prerequisite for a study of this nature is the determination of these equilibrium constants.

The association constants (K_{ass}) for the equilibrium between GSH and the substituted arylglyoxals, as defined by equation (2), at 25 °C in MOPS buffer (0.02M, pH 7.0) were measured

$$K_{ass} = \frac{[ArCO{\cdot}CH(OH)SG]}{[GSH][ArCO{\cdot}CHO]} \quad (2)$$

using the method of Okuyama *et al.*[4] Addition of arylglyoxal to GSH solution at various concentrations in buffer caused an increase in absorbance at λ_{eq}, a wavelength where GSH does not absorb (see Table 1). In this case, equilibrium mixtures were obtained by addition of an aliquot portion of arylglyoxal hydrate (30 μl, 1.3×10^{-2}M; aqueous) to buffer (3.0 ml) containing GSH (0.01—0.1M) at pH 7.0, in a stoppered quartz cuvette thermostatted at 25 °C. The reaction blank used in the spectrophotometer was identical to the reaction solution, except that water was added in place of arylglyoxal solution. The change in absorbance at λ_{eq} with time was followed until the absorbance remained constant.

Table 2. Association constants (25 °C, pH 7.0) for GSH with a series of substituted arylglyoxals

Substituent	σ[a]	K_{ass}/l^{-1} mol	10^3 K_{diss}/l^{-1} mol
H	0.00	1 120 ± 27	0.89
p-Br	0.232	734 ± 15	1.36
p-Cl	0.227	765 ± 26	1.31
p-OCH_3	−0.268	726 ± 36	1.38
p-CH_3	−0.17	894 ± 37	1.12
p-CF_3	0.54	541 ± 18	1.85
p-NO_2	0.778	729 ± 42	1.37
p-C_6H_5	−0.01	981 ± 21	1.02

[a] Taken from ref. 12.

Association constants, k_{ass}, were calculated[4] from the absorbance changes using equation (3) where ΔA is the change

$$\frac{\Delta A}{[GSH]} = K_{ass}\Delta A_{max} - K_{ass}\Delta A \quad (3)$$

in absorbance at λ_{eq} between arylglyoxal alone and arylglyoxal plus GSH; ΔA_{max} is a hypothetical value for the absorbance change when all the arylglyoxal forms hemithioacetal. In these circumstances [GSH] is essentially equal to $[GSH]_0$. Least-squares linear regression treatment of the ΔA/[GSH] *versus* ΔA data gave the association constants k_{ass} as the negative slopes.

Figure 1 shows a typical plot of ΔA/[GSH] *versus* ΔA for *p*-bromophenylglyoxal under these conditions. Values of K_{ass}, along with their inverses ($1/K_{ass} = K_{diss}$), which are the dissociation constants of the respective hemithioacetals, are collected in Table 2 along with some other kinetic data, including Hammett σ values.

Determination of Substrate Properties of Arylglyoxals with Glyoxalase I.—The glyoxalase I-catalysed rearrangement of the hemithioacetals formed between GSH and a series of substituted arylglyoxals [equation (4)] was measured by following the change in absorbance at the apparent isosbestic wavelength (λ_{iso}; Table 1) of the substituted arylglyoxal hydrate and its GSH adduct. In all cases studied, the thiolester products of the glyoxalase I-catalysed reaction have lower molar extinction coefficients at λ_{iso} than the reactants (see Table 1).

A stock solution of substituted arylglyoxal at the required concentration was prepared in DMSO (due to solubility difficulties in water at higher concentrations). Final concentrations of DMSO in the reaction mixture were kept below 1% v/v at which level assay velocities were unaffected by the presence of DMSO. A stock solution of GSH (2.5×10^{-4}M) was prepared in MOPS buffer (0.02M; pH 7.0) and the solution readjusted to pH 7.0 with solid sodium hydrogencarbonate. Human erythrocyte glyoxalase I stock was prepared in the same buffer. GSH stock (3.0 ml) and aliquot portions of arylglyoxal stock (varying in size to give a range of hemithioacetal concentrations) were incubated in a stoppered 1 cm path length quartz cuvette at 25 °C in the thermostatted cell compartment of the spectrophotometer for 8 min to ensure complete hemithioacetal equilibration. Reaction was initiated by the addition of glyoxalase I stock (50 μl). The initial change in absorbance at λ_{iso} with time was measured for a range of hemithioacetal concentrations.

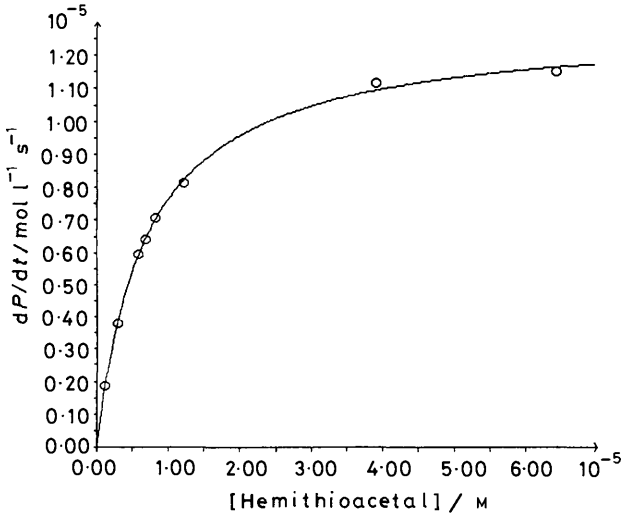


Figure 2. Velocity–substrate profile for the human erythrocyte glyoxalase I-catalysed reaction of the hemithioacetal formed between GSH and *p*-(trifluoromethyl)phenylglyoxal. Points are experimental; line is by nonlinear least-squares regression analysis (see text for details)

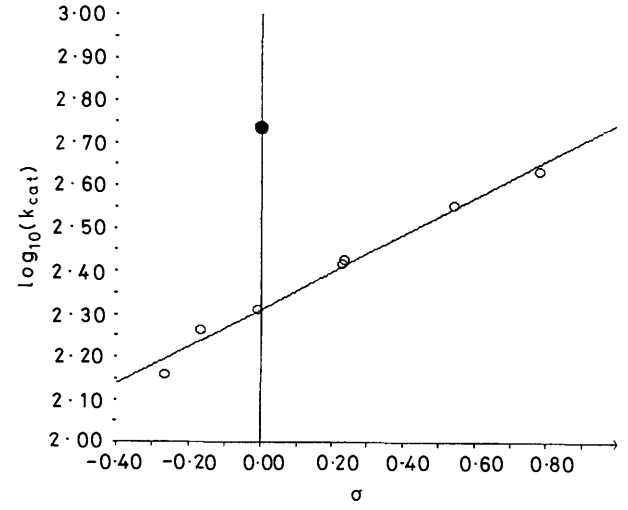


Figure 3. Hammett plot of $\log_{10}(k_{cat})$ *versus* σ constants for the human erythrocyte glyoxalase I-catalysed reaction of the hemithioacetals formed between GSH and a series of *para*-substituted arylglyoxals (○). Datum for the unsubstituted derivative is omitted from the correlation (●). Points are experimental; line is by linear least-squares regression analysis (see text for details)

Hemithioacetal substrate concentrations were determined from the initial concentrations of GSH (determined by Ellman's titration) and arylglyoxal (determined spectrally at $\lambda_{max.}$) in the cuvette, using values for the association constants (K_{ass}). Initial rates (dP/dt) were calculated from equation (4) following the method of Vander Jagt *et al.*,[10] where ΔA_{iso} is the

$$\frac{dP}{dt} = \frac{\Delta A_{iso}}{\Delta t(\varepsilon_s - \varepsilon_p)} \quad (4)$$

initial absorbance change at the isosbestic wavelength in time Δt, and $\varepsilon_s - \varepsilon_p$ is the difference in molar extinction coefficients of hemithioacetal substrate and thiolester product, respectively, at λ_{iso} (values in Table 1).

Plots of initial rate *versus* substrate concentration were fitted by non-linear regression analysis to the Michaelis–Menten

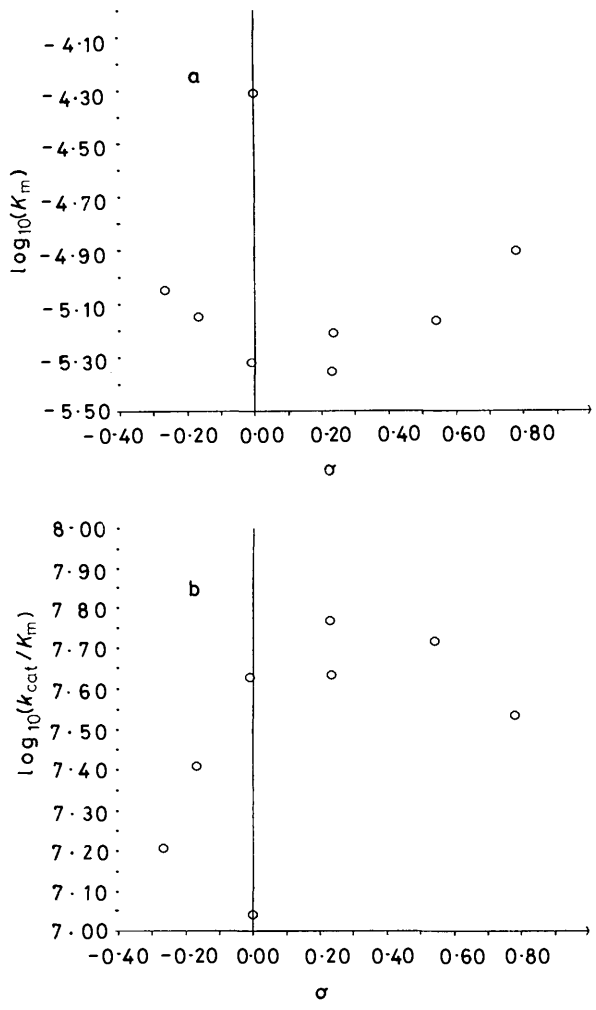


Figure 4. a, Hammett plot of $\log_{10}(K_m)$ *versus* Hammett σ constants for the human erythrocyte glyoxalase I-catalysed reaction of the hemithioacetals formed between GSH and a series of *para*-substituted arylglyoxals. Points are experimental; no significant linear correlation was obtained by linear least-squares regression analysis (see text for details). b, Hammett plot of $\log_{10}(k_{cat}/K_m)$ *versus* Hammett σ constants for the human erythrocyte glyoxalase I-catalysed reaction of the hemithioacetals formed between GSH and a series of *para*-substituted arylglyoxals. Points are experimental; no significant linear correlation was obtained by linear least-squares regression analysis (see text for details)

equation, giving values for the kinetic parameters $V_{max.}$ and K_m for each of the substituted arylglyoxals as glyoxalase I substrates. A typical plot is shown in Figure 2. Values for k_{cat} were obtained using $k_{cat} = V_{max.}/n[E_o]$ where $[E_o]$ is the enzyme concentration, and n is the number of active sites per enzyme molecule ($n = 2$ for human erythrocyte glyoxalase I). A constant concentration (1.8×10^{-8} M) of a single batch of enzyme was used throughout, to ensure comparability of $V_{max.}$ values. Data are summarised in Table 3.

Hammett Analysis of Kinetic Parameters.—A plot of $\log_{10}(k_{cat})$ *versus* Hammett σ values was found to be linear (Figure 3) with slope (ρ) 0.43 (correlation coefficient r 0.984) if the datum for the unsubstituted phenylglyoxal substrate is excluded (see below). The correlation is significantly poorer (r 0.921) if Hammett σ^- values are used with $\log_{10}(k_{cat})$.

Figures 4a and b are Hammett plots for the parameters K_m and k_{cat}/K_m *versus* Hammett σ values, respectively. No linear

Table 3. Kinetic parameters for substituted arylglyoxals as substrates for human erythrocyte glyoxalase I (25 °C and pH 7.0)

$XC_6H_4CO{\cdot}CH(OH)SG$

X	σ[a]	k_{cat}/s^{-1}	$10^6 K_m$/mol l^{-1}	$10^7 k_{cat}K_m^{-1}$/ l mol^{-1} s^{-1}
p-NO_2	0.778[b]	428	12.5 ± 1.1	3.42
p-CF_3	0.54[c]	356	6.85 ± 0.20	5.20
p-Br	0.232	266	6.15 ± 0.79	4.33
p-Cl	0.227	260	4.43 ± 0.35	5.87
H	0.0	540	49.0 ± 0.5	1.10
p-C_6H_5	−0.01	204	4.79 ± 0.25	4.26
p-CH_3	−0.17	184	7.14 ± 0.31	2.58
p-OCH_3	−0.268	144	8.94 ± 1.14	1.61

[a] Hammett σ values *etc.* taken from ref. 12. [b] Hammett σ^- 1.27. [c] Hammett σ^- 0.74.

Table 4. Hammett ρ values for the rearrangement of the hemithioacetals formed between glutathione and substituted-arylglyoxals at 25 °C and pH 7.0 (unless otherwise stated)

Catalyst	Hammatt ρ Value	Parameter Correlated	Reference
Glyoxalase I, human erythrocytes	0.43	k_{cat}	This study
Glyoxalase I, yeast	0.47	$V_{max.}$	10
1,4-Diazabicyclo[2.2.2]octane	0.90	$k_{max.}$	6

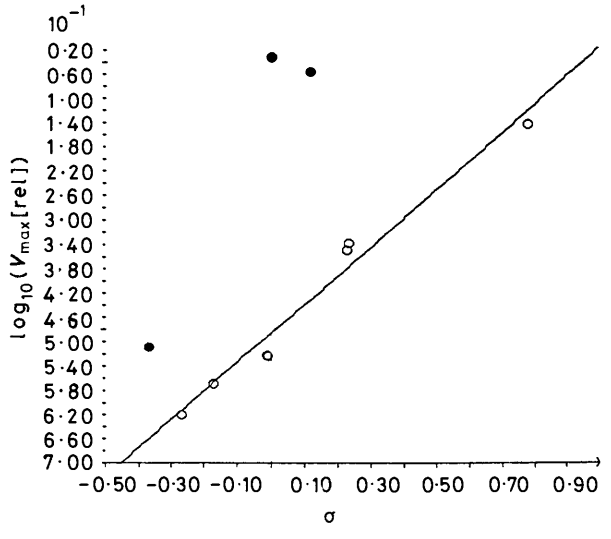


Figure 5. Hammett plot of $\log_{10}$ (relative $V_{max.}$) *versus* Hammett σ constants for the yeast glyoxalase I-catalysed reaction of the hemithioacetals formed between GSH and a series of substituted arylglyoxals (○). Data for some derivatives are omitted from the correlation (●). Points are experimental; line is by least-squares linear regression analysis (see text for details). Data from ref. 10

Hammett correlation is observed in either case. Correlation coefficients, obtained by least-squares linear regression analysis, were *r* 0.001 for the K_m data, and *r* 0.282 for k_{cat}/K_m. The Hammett equations (and correlation coefficients *r*) for the various parameters (k_{cat}, K_m, k_{cat}/K_m) are given in equations (5)—(10).

$$\log_{10}(k_{cat}) = 0.43(\pm 0.02)\sigma + 2.31\ (r\ 0.984) \quad (5)$$

$$\log_{10}(k_{cat}) = 0.29(\pm 0.04)\sigma^- + 2.31\ (r\ 0.921) \quad (6)$$

$$\log_{10}(K_m) = -0.03(\pm 0.38)\sigma - 5.05\ (r\ 0.001) \quad (7)$$

$$\log_{10}(K_m) = 0.02(\pm 0.26)\sigma^- - 5.06\ (r\ 0.01) \quad (8)$$

$$\log_{10}(k_{cat}/K_m) = 0.38(\pm 0.25)\sigma + 7.43\ (r\ 0.282) \quad (9)$$

$$\log_{10}(k_{cat}/K_m) = 0.22(\pm 0.18)\sigma^- + 7.44\ (r\ 0.191) \quad (10)$$

The equations for k_{cat} were obtained by omitting the datum for the unsubstituted phenylglyoxal (X = H) substrate. The correlation for k_{cat} with σ values is significantly decreased (*r* 0.42) if the X = H derivative is included.

Discussion

It is clear from Figure 3 and equations (5)—(10) that the only good linear Hammett correlation is obtained for human erythrocyte glyoxalase I when k_{cat} is considered. For this the ρ value is 0.43. There are other literature data available for this reaction and we have undertaken Hammett analysis of these. The results are summarised in Table 4. For the reaction of equation (1), catalysed by glyoxalase I from yeast (*Saccharomyces cerevisiae*),[10] if the values of the kinetic parameters obtained for the unsubstituted (X = H), the *m*-methoxy, and the *p*-hydroxy derivatives are omitted, relative $V_{max.}$ values correlate with Hammett σ values according to equation (11). In

$$\log_{10}(V^{rel}_{max.})\ \text{yeast} = 0.47(\pm 0.04)\sigma - 0.49\ (r\ 0.96) \quad (11)$$

this case no correlation with σ values is seen for $\log_{10} K_m$ (*r* 0.002), or for $\log_{10}(V_{max.}/K_m)$ (*r* 0.254).

The *p*-hydroxy derivative is omitted due to the difficulty in accurately assigning a Hammett σ value to the hydroxy group under study at a pH approaching its pK_a in the free substrate. The *p*-hydroxy group of the parent acid of *p*-hydroxyphenylglyoxal can be estimated[11] to have pK_a *ca.* 8.0. At pH 7.0,[10] where a substantial proportion (in the region of 10%) of the *p*-hydroxy groups would be present as the anionic (O^-) form, the Hammett σ value we should use is a linear combination of those for *p*-OH (−0.37)[12] and *p*-O^- (−1.00),[12] which gives a value of −0.43. In addition, σ values for charged groups may be particularly solvent-dependent. Finally, the situation for the enzyme-bound substrate *p*-OH group may not be fairly reflected by the pK_a for the free substrate *p*-OH.

There are also data for rat erythrocyte glyoxalase I catalysing this reaction[13] but if the *p*-OH compound is excluded the range of σ values involved (from −0.27 to +0.23) is too small to allow dependable Hammett analysis.

It is curious that the unsubstituted phenylglyoxal-derived hemithioacetal data are not correlated by the Hammett analyses for either human erythrocyte or yeast glyoxalase I. It is not obvious why this should be so, but a deviation of a rather similar nature for the unsubstituted phenyl member of a range of aryl mesylglycinate esters as substrates for papain has been reported.[14] In the case of glyoxalase I the hydrogen substituent appears to have a rate enhancement of *ca.* 2.5-fold for the yeast and human erythrocyte enzymes, respectively. For the papain case, on the other hand, the *p*-H group causes a rate *decrease* of *ca.* 3-fold. In glyoxalase I, it could be that part of the function of the *para*-substituent is a binding feature reflected in k_{cat}. Multiple productive-binding modes may be possible for the *p*-H system but not for the other substituents, the *para*-groups of which may 'lock' substrate binding into fewer correct (*i.e.* productive) orientations.

The need to exclude the *m*-methoxy point from the corre-

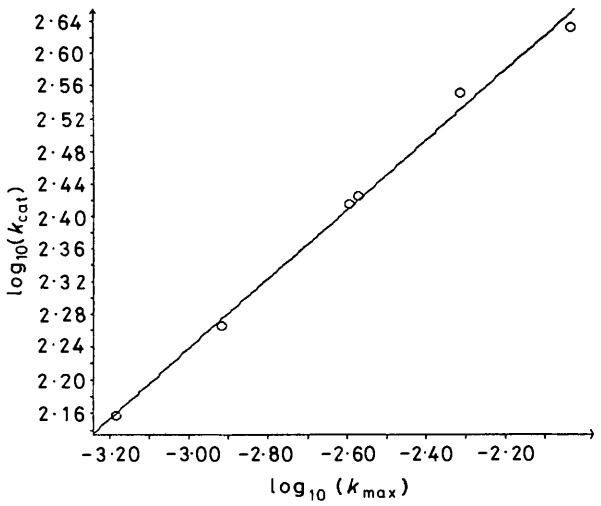


Figure 6. Plot of $\log_{10}(k_{cat})$ for the human erythrocyte glyoxalase I-catalysed reaction of the hemithioacetals formed from GSH and a series of substituted arylglyoxals *versus* $\log_{10}(k_{max.})$ for the DABCO-catalysed model system utilising the same series of arylglyoxals. Points are experimental; line is by least-squares regression analysis (see text for details). Data for k_{cat} from this study; data for $k_{max.}$ from ref. 6

lation for yeast glyoxalase I needs some explanation. It may be that a *meta*-substituted substrate series would behave differently in the linear free energy relationship with the enzyme than their *para*-counterparts. A dispersion of a Hammett plot into *meta*- and *para*-'families' (with low but clearly distinct correlations) has been reported for cyclodextrin-catalysed hydrolyses of substituted phenyl acetates.[15] Whilst electronically *para*- and *meta*-'families' of substituents are usually identical in physical organic terms, when host–guest situations arise (as in inclusion complexes such as the cyclodextrins or in enzyme active sites) they need not be. Therefore, *meta*-substituted aryl groups could clearly be distinct from *para*-substituted aryl groups at the interface with an enzymic recognition-site.

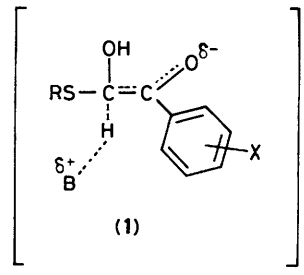
Comparison with Model Systems.—Model studies in the absence of glyoxalase I suggested[4–6,16] that the rearrangement of hemithioacetals formed from phenylglyoxal and thiols to corresponding thiolesters proceeds *via* a proton-transfer mechanism. Okuyama *et al.*[4] used the saturation-dependence of rate on thiol concentration to demonstrate the participation of the hemithioacetal in the reaction pathway [equation (12)].

$$\begin{matrix} RSH \\ + \\ ArCOCHO \end{matrix} \overset{K_h}{\rightleftharpoons} ArCOCH(OH)SR \xrightarrow{k_{max}} ArCH(OH)CO{\cdot}SR \quad (12)$$

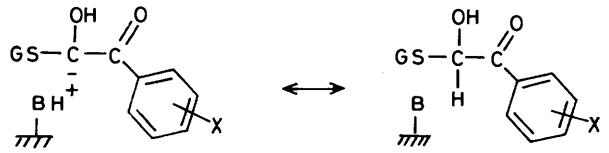
Formation constants (K_h) for hemithioacetalisation were identical when measured by both kinetic and equilibrium approaches. The $k_{max.}$ step for the model system refers to the rearrangement,[6] and describes general base-catalysis[4] for this step.

In a recent model study of the glyoxalase I catalysed reaction, the DABCO-catalysed rearrangement of hemithioacetals formed from 2-mercaptoethanol and a series of substituted arylglyoxals was studied kinetically.[6] It was found that $\log_{10} k_{max.}$ for DABCO catalysis of this reaction showed a fit to Hammett σ values rather than σ^- values (see Table 4), indicating that resonance stabilisation of the transition state by the aryl substituent does not occur.

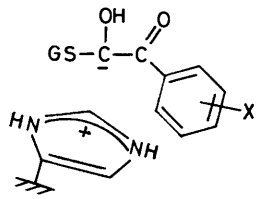
In model systems the most clearcut interpretation of the data is that deprotonation at C(1) (*i.e.* the carbon atom α to the sulphur) is the rate-determining step for the general base-catalysed rearrangement of the hemithioacetals; the proposed transition-state structure is (**1**).



(**1**)



(**2**) (**3**)



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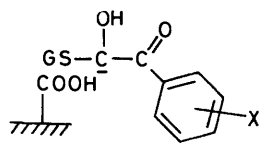

(**5**)

In the DABCO-catalysed model studies, $k_{max.}$ reflects a comparatively simple rearrangement, whereas the enzymic parameter (k_{cat}) could include contributions from other features, such as binding and/or conformational change(s) at the active site of the enzyme. Figure 6 shows that there is a strong correlation (r 0.99) between values for $\log_{10} k_{cat}$ from the present human erythrocyte glyoxalase I study. This indicates that both the DABCO- and glyoxalase I-catalysed hemithioacetal rearrangements are similarly sensitive to substituent effects and that they may follow very similar mechanisms, *i.e.* probably *via* the rate-determining deprotonation at C(1) (**1**). However, the lower ρ value obtained in the glyoxalase I-catalysed study requires some examination.

In order to explain the lower ρ value obtained in the enzymic case, two hypothetical extremes of the transition state (**1**) can be considered, (**2**) and (**3**).

In (**2**), complete deprotonation at C(1) has occurred, and the resulting negative charge will be stabilised by electron-withdrawing substituents X. The other extreme, (**3**), where no deprotonation at all at C(1) has taken place, will not be influenced by electron-withdrawing groups at X to anything like the same extent. Hammett ρ values would be expected to increase as the structure of the transition state approaches (**2**) in character, relative to (**3**). The ρ value obtained for the human erythrocyte and yeast glyoxalase I-catalysed reactions (*ca.* 0.4—0.5) is lower than the value obtained for the model reaction (ρ +0.90), which might indicate that the enzymic transition state shows less C(1)–H cleavage than the model.

However, this is a highly simplified argument. A lower or changed ρ value in an enzymic compared to a model system is not uncommon, and can arise from a number of factors peculiar to enzymes. The environment surrounding the substrate at the active site is crucial. A lowering of the ρ value in this (glyoxalase)

case could result from local stabilisation of the negative charge at C(1) by neighbouring group(s) at or near the active site, *e.g.* electrophilic assistance,[17] from the active-site zinc atom or from a hydrogen-bond. Electrophilic effects of this type have been discussed by Williams for α-chymotrypsin[17] and papain.[14]

An extra possibility not previously discussed to our knowledge, is the nature of the active-site base which probably removes the proton from C(1) and could exert a profound stabilising effect on any negative charge at C(1). If this active-site base were a neutral base (*i.e.* $B + H^+ \rightleftharpoons BH^+$), then the negative charge would be stabilised by the forming, neighbouring partial positive charge resulting from protonation of the base (**4**) in the transition state although the charge localisation on the ring nitrogen would weaken the effect because of the distances involved. This case would not arise if the active-site base were negative (*i.e.* $B^- + H^+ \rightleftharpoons BH$), since no positive charge would result, (**5**), on it from proton transfer. In the model system studies,[6] the rearrangement of the hemithioacetal was catalysed by DABCO, essentially a neutral base at its attacking nitrogen end, which will facilitate the stabilisation of the negative charge at C(1) on deprotonation. In any case in free aqueous solution such an electrostatic effect will be minimised because of the high dielectric constants.

As yet, the nature of the functional active-site base in human erythrocyte or yeast glyoxalase I is unknown, so no conclusions can be drawn as to whether it would help stabilise the transition-state, and thus reduce the value of ρ compared to the model system. Certainly, such an effect is more likely to be of significant magnitude in a local, low dielectric region on an enzyme than in bulk water.

The ρ values for the glyoxalase I-catalysed rearrangements of the hemithioacetal substrates (ρ 0.43—0.47) are somewhat lower than the value for the model (amine-catalysed) rearrangement[6] (ρ +0.90), but probably the mechanisms are grossly similar. Some further light can be shed on this by consideration of primary isotope effects on $V_{max.}$ for the glyoxalase I-catalysed and $k_{max.}$ for the DABCO-catalysed rearrangements.

Values of k_H/k_D

2.9 for R = CH_3
3.2 for R = C_6H_5 for $V_{max.}$ (yeast glyoxalase I)[18]

1.7 for R = CH_3
4.8 for R = C_6H_5 for $V_{max.}$ (rat glyoxalase I)[13]

5.9 for R = C_6H_5 for $k_{max.}$ (model system)[6]

The k_H/k_D value for the model system is considerably greater than for the enzyme system but is still not at the theoretical limit of *ca.* 7. A lowered value of k_H/k_D (leaving aside the inevitable complications of any enzymic case) could simply indicate either a transition state with markedly *reduced* proton transfer, or markedly *increased* proton transfer, because of the 'bell-shaped' relationship reported between k_H/k_D and the extent of proton transfer.[19] It is not totally safe to decide in favour of either of these possibilities because the $k_{max.}$ values obtained in the model system[6] are less likely to be kinetically complex than the k_{cat} values of the enzyme system with which they are compared, *e.g.* the enzymic k_{cat} may reflect features such as binding, conformational changes, *etc.* However, a referee has suggested that the lowered ρ values, taken along with the lower *k*s indicates that there is less proton transfer in the enzymatic case.

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References

1 K. T. Douglas and S. Shinkai, *Angew. Chem., Int. Ed. Engl.*, 1985, **24**, 31.
2 S. J. Carrington and K. T. Douglas, *IRCS Med. Sci.*, 1986, **14**, 763.
3 K. T. Douglas, D. I. Gohel, I. N. Nadvi, A. J. Quilter, and A. P. Seddon, *Biochim. Biophys. Acta*, 1985, **829**, 109.
4 T. Okuyama, K. Kimura, and T. Fueno, *Bull. Chem. Soc. Jpn.*, 1982, **55**, 1493.
5 S. Shinkai, T. Yamashita, Y. Kusano, and O. Manabe, *J. Am. Chem. Soc.*, 1981, **103**, 2070.
6 K. T. Douglas and H. Demircioglu, *J. Chem. Soc., Perkin Trans. 2*, 1985, 1951.
7 A.-C. Aronsson, G. Tibbelin, and B. Mannervik, *Anal. Biochem.*, 1979, **92**, 390.
8 P. W. Riddles, R. L. Blakeley, and B. Zerner, *Meth. Enzymol.*, 1983, **91**, 49.
9 H. A. Riley and A. R. Gray, *Org. Synth.*, 1943, Coll. Vol. II, 509.
10 D. L. Vander Jagt, L.-P. B. Han, and C. H. Lehmann, *Biochemistry*, 1972, **11**, 3735.
11 Estimate based on 'Handbook of Biochemistry and Molecular Biology, Physical and Chemical Data,' ed. G. D. Fasman, C.R.C. Press, Cleveland, 1976, 3rd edn., vol. 1, p. 314.
12 J. Hine, 'Physical Organic Chemistry,' McGraw-Hill, New York, 1962, p. 87.
13 L.-P. B. Han, L. M. Davison, and D. L. Vander Jagt, *Biochim. Biophys. Acta*, 1976, **445**, 486.
14 A. Williams, E. C. Lucas, and A. R. Rimmer, *J. Chem. Soc., Perkin Trans. 2*, 1972, 621.
15 R. L. Van Etten, J. F. Sebastian, G. A. Clowes, and M. L. Bender, *J. Am. Chem. Soc.*, 1967, **89**, 3242.
16 S. S. Hall, A. M. Doweyko, and F. Jordan, *J. Am. Chem. Soc.*, 1976, **98**, 7960.
17 A. Williams, *Biochemistry*, 1970, **9**, 3383.
18 D. L. Vander Jagt and L.-P. B. Han, *Biochemistry*, 1973, **12**, 5161.
19 R. P. Bell, 'The Proton in Chemistry,' Chapman and Hall, London, 1974, p, 264.

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