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THE MEISENHEIMER COMPLEX OF GLUTATHIONE AND TRINITROBENZENE

A POTENT INHIBITOR OF THE GLUTATHIONE *S*-TRANSFERASE FROM *GALLERIA MELLONELLA*

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Abstract—1. The Meisenheimer complex formed between reduced glutathione and 1,3,5-trinitrobenzene is characterised by an extinction coefficient at 470 nm of 20400 and by an association constant at pH 9.18 of 42 $l \cdot mol^{-1}$.

2. Trinitrobenzene is a moderately good inhibitor of the glutathione *S*-transferase from larvae of the moth *Galleria mellonella*. It acts by competition with the electrophilic substrate. At pH 7.4, it has a K_i value of 10 μM. Its mode of inhibition with respect to GSH† appears to be non-competitive.

3. At pH values below 9.0, the Meisenheimer complex does not appear to be formed in sufficient quantity to give significant inhibition of the enzyme. At pH 9.0 and at GSH concentrations greater than 1 mM, the inhibition of the enzyme became markedly non-hyperbolic. This was attributed to the inhibitory action of the Meisenheimer complex. The complex appears to act also by competition with the electrophilic substrate and its K_i is calculated to be 1.7×10^{-7} M.

The glutathione *S*-transferases play a major role in the detoxification of electrophilic molecules of both endogenous and exogenous origin [1]. Of particular interest has been the role that they may play in the development of tolerance to such compounds. Thus tolerance of strains of cancer cells to antineoplastic drugs has been related to cellular concentrations of glutathione and the glutathione transferases [2–6], and it has become increasingly clear that the development of pesticide resistance in plant and arthropod pests may depend strongly on the activity of these enzymes [7–12].

Given such considerations, the ability to inhibit these enzymes *in vivo* might play an important role in modulating aspects of intermediary metabolism [13–17] or of countering the phenomenon of pharmacological tolerance. We have thus recently turned to an examination of classes of compound which might serve as inhibitors of these enzymes *in vivo*.

In the present work, we examine inhibition of the enzymes by 1,3,5-trinitrobenzene. This has been shown to form a Meisenheimer complex with glutathione [18]. Such complexes, resembling the frequently encountered *S*-substituted GSH conjugates, could well be expected to be inhibitory in the first instance because of their resemblance to the reaction products of the enzyme catalyzed reaction.

MATERIALS AND METHODS

Materials

Reduced glutathione was purchased from Sigma Chemical Co (St Louis, MO) and 1-chloro-2,4-dinitrobenzene from BDH Chemicals Ltd (Poole, Dorset, U.K.). Picric acid (2,4,6-trichlorophenol) was obtained from Ajax Chemicals (Sydney, Australia).

Picryl chloride (m.p. 82°) was synthesized from picric acid by the method of Boyer *et al.* [19] and trinitrobenzene (m.p. 120–122°) from picryl chloride by the method of Read *et al.* [20]. Trinitrophenetole (m.p. 77–79°) was synthesized by the method of Brady and Horton [21] and 2,4-dinitrophenetole (m.p. 85°) and 2,4-dinitroanisole (m.p. 81–83°) were synthesized by the same method.

Glutathione *S*-transferase from *Galleria mellonella* was produced by combined affinity chromatography and ion exchange as described previously [22]. It was homogeneous by the criteria of SDS–PAGE and analytical isoelectrofocusing.

Methods

Characterization of Meisenheimer complex formation. A stock solution of 1,3,5-trinitrobenzene (1.3 mM) was prepared by dissolving the solid compound in 0.01 M sodium borate buffer, pH 9.18 (Buffer A). A stock solution of GSH of 0.3 M in Buffer A, readjusted to pH 9.18 with 2 M NaOH solution was used. These stock solutions were diluted into Buffer A to give, in a total volume of 3 ml, concentrations of TNB ranging from 0.05 to 0.2 mM and GSH concentrations from 1 to 50 mM. Formation of the Meisenheimer complex was indicated by the appearance of a red colour in the mixture, corresponding with an increase in extinction at 470 mm [18]. The increase in extinction was measured in a Pye-Unicam SP8-400 double-beam spectrophotometer. Measurements were made as quickly as possible after mixing, because of the sub-

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† Abbreviations used: GSH: reduced glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; TNB, 1,3,5-trinitrobenzene.

sequent slow decay of the complex [18]. Readings were made in triplicate. Mean values thus obtained were fitted to Eqn. 1, to give estimates of the value of the association constant (K_a) for the formation of the complex E_m, the molar extinction coefficient for the complex.

$$E = E_m \frac{\left\{([GS^-] + [TNB] + K_a^{-1}) - \left[([GS^-] + [TNB] + K_a^{-1})^2 - 4.[GS^-].[TNB]\right]^{1/2}\right\}}{2} \quad (1)$$

The fitting procedure was carried out by using a non-linear least-squares regression program, KINETICS 301, written for the Apple IIe computer. The regression was unweighted.

Enzyme kinetic studies. The effects of TNB (and also picric acid) on the catalytic reaction of the glutathione *S*-transferase were studied at three pH values, 6.5, 7.4 and 9.0. Buffers used were 0.1 M sodium phosphate at the two lower pH values and 0.025 M sodium borate at pH 9.0. CDNB concentrations were varied between 0.1 and 0.9 mM (GSH held constant at 1 mM), appropriate dilutions being made from a stock solution of CDNB (1.0 mM) dissolved directly in the assay buffer. Glutathione concentrations were varied (at a fixed CDNB concentration of 0.5 mM) between 0.2 and 4 mM, dilution being made from a stock solution adjusted to the assay pH. Inhibition by trinitrobenzene (and by picric acid) was studied by dissolving the compound directly in the assay buffer at the desired concentration. Concentrations of 0.05 and 0.1 mM were used in the case of TNB.

The above type of experiment was repeated with 0.1 M borate buffer (pH 9.2) to improve pH control and also at 15°, to reduce the magnitude of the spontaneous reaction.

The enzymic reactions were initiated by adding purified glutathione *S*-transferase to the assay mixture. The increase in extinction at 344 nm was monitored using a Varian-Cary 210 spectrophotometer. The temperature was maintained at 25°. Measurements of reaction rates were made in triplicate. They were corrected for the spontaneous rate where appropriate. The corrected rates were fitted to selected equations by the non-linear regression routine discussed above.

In studying the variation with pH of the effect of trinitrobenzene on the enzyme-catalysed reaction, CDNB (0.1 mM) was chosen as substrate. The GSH concentration was either 1 or 4 mM. Buffers used were: pH 7.0–8.0, 0.1 M sodium phosphate; pH 7.5–9.0, 0.1 M Tris–HCl and pH 8.5–10.0, 0.025 M sodium borate. Reaction rates were measured as described above.

RESULTS

Formation of Meisenheimer complex

The association of GSH and TNB to form a Meisenheimer complex was examined quantitatively at pH 9.18. The association constant between the aromatic compound and the thiolate anion of GSH was determined to be 42 ± 7 $l \cdot mol^{-1}$ and the molar extinction coefficient to be 20,400 ± 1300. Data were obtained at six concentrations of glutathione and seven concentrations of TNB. Points were determined in quintuplicate.

An additional experiment was carried out using a higher concentration buffer (0.1 M) to achieve greater control of pH values. The results in this case were not significantly different from above.

This type of experiment was also carried out at pH 6.5 and pH 7.4. There was no detectable formation of the adduct under these conditions.

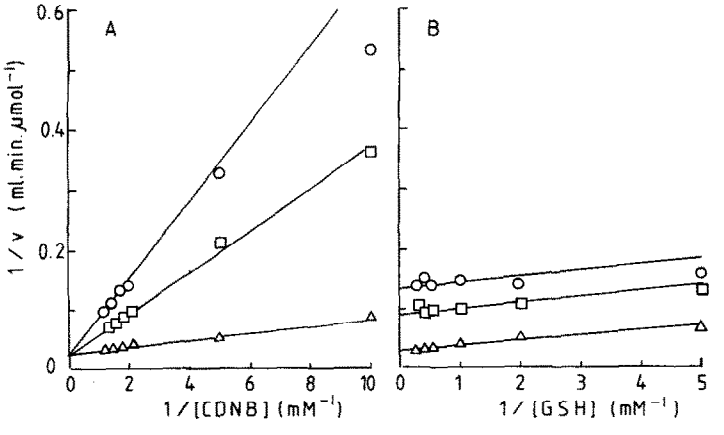

Fig. 1. Inhibition by 1,3,5-trinitrobenzene of glutathione *S*-transferase from *Galleria mellonella* at pH 7.4. Enzyme catalysed reaction rates were determined in the absence (△) and in the presence of 0.05 mM (□) and 0.1 mM (○) TNB. The data are presented as double reciprocal plots. The experimental points shown are the means of at least quadruplicate determination. Coefficients of variation on these means were less than 0.15. Solid lines are lines of best fit calculated by least squares regression. Regressions were carried out on the data sets in A and B independently.

Table 1. Variation with pH of apparent kinetic constants pertaining to the inhibition of glutathione *S*-transferase by 1,3,5-trinitrobenzene

Varied substrate	pH	Parameters		
		V_m (μmol/min)	K_m (mM)	K_i (mM)
GSH	6.5	14.7 ± 0.4	0.33 ± 0.02	0.06 ± 0.007
	7.4	31.8 ± 1.6	0.22 ± 0.04	0.028 ± 0.002
CDNB	6.5	28.0 ± 3.7	0.82 ± 0.16	0.057 ± 0.007
	7.4	34.8 ± 2.1	0.19 ± 0.03	0.01 ± 0.001

Results are best-fit regression parameters ± SD. Regressions were performed on data sets of 18 points, each of which was the mean of at least four determinations.

Effects of TNB on the enzyme catalyzed reaction

In initial experiments at pH 6.5 and 7.4 TNB was found to be a quite powerful inhibitor of the *Galleria* glutathione *S*-transferase. It was found to act as a competitor with the electrophilic substrate, CDNB. When GSH was the varied substrate, a model of uncompetitive inhibition gave the best fit to the experimental data (see Fig. 1). These results were qualitatively consistent over this pH range. There was quantitative variation in the results obtained at different pH values (see Table 1) but the compound TNB shown to be a good inhibitor, apparent K_i values going as low as 7 μM, depending on the experimental conditions. There was no strong evidence in these experiments that TNB was acting in any fashion other than as an unreactive analogue of CDNB.

However, the experiment was also carried out at pH 9.0. This produced a striking qualitative change in the inhibition of the enzyme (Fig. 2). Higher concentrations of GSH led to a marked increase in the degree of inhibition. This is not an effect due to GSH alone since there was no evidence of substrate inhibition in the absence of TNB. Inhibition with respect to CDNB still appeared to be competitive. The non-linear inhibition with respect to GSH seen at high pH was seen also when 0.1 M borate buffer was employed and also when the reaction temperature was reduced to 15°. This change in the mode of inhibition at high pH was interpreted as being due to the formation of a strongly inhibitory Meisenheimer complex.

This was tested by fitting the data in Fig. 2 to each of eight equations that might account for inhibition by both TNB and the adduct. The equation giving the best fit to the data was Eqn 2, below:

$$v = \frac{V}{1 + \frac{K_g}{[GSH]} + \frac{K_d}{[CDNB]} \cdot \left(1 + \frac{[TNB]}{K_t} + \frac{[MC]}{K_c}\right) + \frac{K_g . K_d}{[GSH][CDNB]} \left(1 + \frac{[TNB]}{K_t} + \frac{[MC]}{K_c}\right)} \quad (2)$$

In this equation V is the limiting velocity, K_g and K_d are Michaelis constants with respect to GSH and CDNB respectively, and K_t and K_c are inhibition constants with respect to TNB and the Meisenheimer complex (MC) respectively. In fitting data to this equation, the concentrations of [MC] were calculated

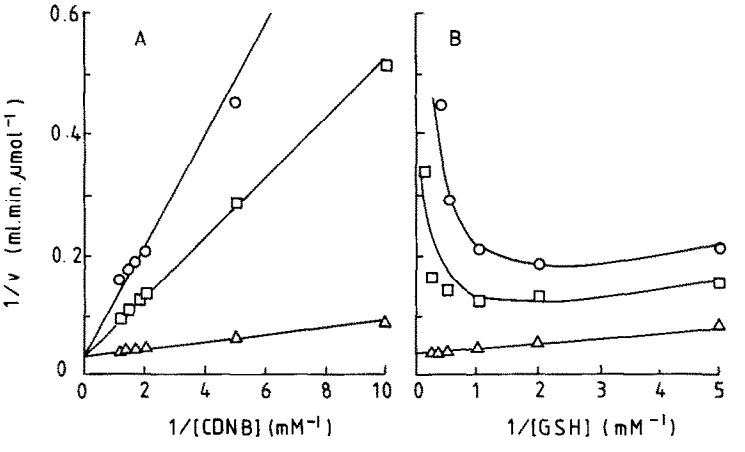

Fig. 2. Inhibition by 1,3,5-trinitrobenzene of glutathione *S*-transferase from *Galleria mellonella* at pH 9.0. Symbols as in Fig. 1. Coefficients of variation on the quintuplicate rate determinations fell into the range 0–0.20. The mean value was 0.13. Solid lines are lines of best fit to the whole data set included in both A and B.

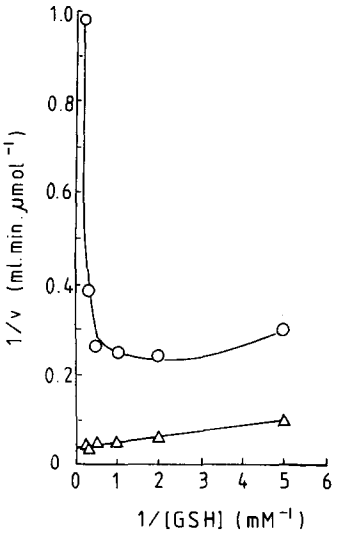

Fig. 3. Inhibition by 2,4,6-trichlorophenol of glutathione *S*-transferase from *Galleria mellonella* at pH 9.0. Enzyme catalysed reaction rates were measured in the presence (○) of 0.05 mM picric acid, and in its absence (△). Data points were determined in triplicate.

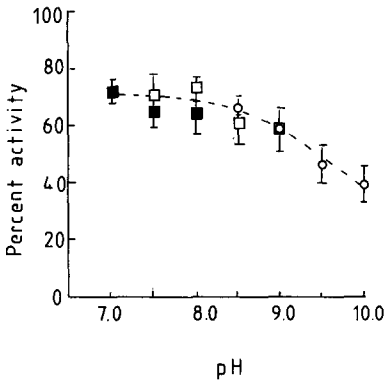

Fig. 4. Inhibition of glutathione *S*-transferase from *Galleria mellonella* by 1,3,5-trinitrobenzene and its variation with pH. Assays were carried out at the indicated pH using 4 mM and 0.1 mM CDNB as described in Methods. Buffers used were: ■, 0.1 M sodium phosphate; □, 0.1 M Tris–HCl; ○, 0.025 M sodium borate. Results show the reaction velocity determined in the presence of the inhibitor as a percentage of the uninhibited reaction rate at indicated pH.

from [GSH] and [TNB] using Eqn 1. They reached values as high as 1.6 μM. The velocities were unweighted for the fitting procedure [23]. The values obtained by regression were: $V = 34.5 \pm 1.2$ μmol/min/ml; $K_d = 0.15 \pm 0.01$ mM; $K_g = 0.23 \pm 0.026$ mM; $K_t = 0.017 \pm 0.004$ mM; $K_c = 0.17 \pm 0.027$ μM.

The equation finally chosen corresponds to a mechanism in which both TNB and the Meisenheimer complex compete with CDNB and act non-competitively (although generating near-parallel, and hence apparently uncompetitive, double-reciprocal plots) with respect to GSH.

It was expected that if this inhibition were due to formation of an adduct then increasing the assay pH should increase the degree of inhibition observed. This should result from the increasing concentration of the glutathione sulphydryl anion and hence of the equilibrium concentration of the adduct. To test this, enzyme activity, assayed with 0.1 mM CDNB and 1 or 4 mM GSH, was examined over the pH range 7.0–10.0, in the presence and absence of 0.01 mM TNB. This led to an approximately constant inhibition (30%) over the whole pH range when GSH was maintained at 1.0 mM (not shown). This is consistent with the results obtained with the detailed kinetic studies, summarized in Table 1, in which it is seen that the ratio K_d:K_t is approximately constant over the whole range. On the other hand, when GSH concentration was increased to 4 mM, a rather marked increase in inhibition was seen when the pH rose above 8.5. These data are consistent with the formation of a strongly inhibitory Meisenheimer complex. Under the latter conditions, the concentration of the Meisenheimer complex at pH 9.0 was calculated to be 0.64 μM. The extent of inhibition predicted from Eqn 2 is therefore approximately 70% of which 26% inhibition is accounted for by the uncomplexed TNB.

The data shown in Fig. 1(b) do diverge somewhat from the lines predicted by a model involving simple uncompetitive inhibition by TNB. One referee suggested that this behaviour might be accounted for by inhibition by traces of the complex even at pH 7.4. We have examined this possibility. At this pH, concentrations of the complex of up to 2.7×10^{-7} M would be expected. Fitting the data to an equation derived from Eqn 2 (allowing for inhibition by the complex) did improve the fit of the data to theoretical curves. However, the value for K_c obtained ($4.0 \pm 2.4 \times 10^{-8}$ M) was poorly defined (coefficient of variation = 0.6). We interpret our data to suggest that inhibition of this sort may take place at pH 7.4, but that the effect is not marked and cannot be considered to have been unequivocally demonstrated.

The effects seen with TNB were not detected with m-dinitrobenzene, dinitroanisole and 3,4-dichloronitrobenzene. When these compounds were tested at 0.05 and 0.10 mM and (for DCNB) 0.4 mM, there was no evidence of significant Meisenheimer complex formation, and no inhibition was seen. On the other hand, picric acid at 0.05 and 0.1 mM proved to be strongly inhibitory (apparent K_i of the order of 10–30 μM) at pHs between 6.5 and 9.2. At pH 9.0, but not at the lower pHs, the mode of inhibition was again markedly non-linear (Fig. 3) and again, it is suggested that a strongly inhibitory Meisenheimer complex is formed. The strong extinction at the assay wavelength of the solutions of picric acid used made rate determinations difficult, so that detailed mechanistic studies have not been undertaken with this compound.

DISCUSSION

In the experimental work recounted here we have evidence that the Meisenheimer complexes formed between the aromatic nitro compounds 1,3,5-trinitrobenzene and picric acid, and glutathione, are potent inhibitors of a glutathione *S*-transferase from

Galleria mellonella. These complexes, which may be regarded as *S*-modified glutathiones, appear to be more potent inhibitors of the enzyme than any other glutathione *S*-conjugate yet studied. The reason for this may be that these complexes may resemble the transition state for the reaction in which glutathione *S*-conjugates are formed by nucleophilic attack by GS^- at an electrophilic centre on the substrate (see Refs 22 and 24). If the enzyme catalyzed reaction proceeds through an SN (aromatic) displacement reaction, then such a transition state analogue might be expected to be a potent inhibitor of the reaction [25].

The TNB–GSH complex appears to be a very potent inhibitor of the enzyme. The K_i of 0.17 μM is over 200× less than the K_i reported for this enzyme for the closely related conjugate *S*-2,4-dinitrophenyl-L-glutathione [26]. This tends to support the suggestion that this complex is not acting merely as glutathione conjugate. It may, however, be premature to suggest that it is acting as a true transition state analogue. On the basis of the Michaelis constants for GSH and CDNB, one might expect even tighter binding from the true transition state analogue. Given that the mechanism for the *Galleria* glutathione *S*-transferases is probably random bi-bi [26], then the expected inhibition from such analogues would be competitive with respect to both substrates. The non-competitive inhibition with respect to GSH was not anticipated and casts doubts on whether the complex is in fact acting as an analogue of the transition state rather than merely as a reaction intermediate.

It should be stressed that the interpretation we place on our experimental data is a tentative one. At the high pH values at which complex formation is significant, the spontaneous reaction is rapid, which necessarily reduces the precision of measurement of the catalyzed reaction rates. Mechanistic interpretations of the kinetic data are thus made with caution. However, the sum of our data does suggest that the Meisenheimer complexes studied in this work are potent inhibitors of the glutathione transferase.

The present work is essentially an academic study in that, with trinitrobenzene as precursor, unphysiologically high pH values are used for the generation of inhibitory amounts of adduct. However, Meisenheimer complexes are formed from a wide variety of chemicals and other compounds may form inhibitory adducts at physiological pHs. A class of compound which might repay examination is the benzofurazan type. These may form Meisenheimer complexes under mild conditions (see Ref. 27) with cellular nucleophiles. Some, particularly 4- and 5-nitro benzofurazans, are known to react with GSH at pH 7.4 and may then generate products capable of potent inhibition of the glutathione *S*-transferase under physiological conditions.

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