Species Differences in the Inhibition of Glutathione *S*-Aryltransferase by Phthaleins and Dicarboxylic Acids

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1. Glutathione-*S*-aryltransferase activity from grass grubs (*Costelytra zealandica*) was inhibited by phthaleins, sulphonphthaleins and some dicarboxylic acids. 2. These compounds had no detectable action on the enzyme from sheep liver. 3. In insect enzyme the inhibition was competitive with respect to glutathione and non-competitive with respect to the aromatic substrate. 4. Michaelis constants and inhibitor constants were measured for sheep-liver or grass-grub enzyme between pH 5 and pH 10 and evidence was obtained for the presence of two groups with p*K* 9.2 in the glutathione-binding site of the insect enzyme. 5. Only one such group was detected in the sheep-liver enzyme.

Differences between the insect and vertebrate glutathione *S*-aryltransferases were reported by Cohen, Smith & Turbert (1964), who found that bromsulphalein was a poor substrate for the enzyme from locusts. In vertebrates the analogous enzyme shows sufficient activity with this substrate for its metabolism to be the basis of the well-known liver-function test (Combes & Stakelum, 1961). Bromsulphalein was an effective inhibitor of the enzyme from several insect species and showed non-competitive kinetics with respect to DCNB* (Cohen *et al.* 1964). With rat enzyme, however, only a small degree of inhibition by bromsulphalein was found by Grover & Sims (1964) and this was competitive with respect to the aromatic substrate.

The metabolism of gammexane (γ -1,2,3,4,5,6-hexachlorocyclohexane) in insects gives rise to *S*-conjugates (Bradbury & Standen, 1959) and probably involves a transferase enzyme of this type since a glutathione derivative is a major metabolite (Clark, Hitchcock & Smith, 1966). In vertebrates this type of metabolite is less important and phenols are the major end products of Gammexane metabolism (Grover & Sims 1965). This species difference in the detoxication of a widely used insecticide and the apparent importance of glutathione transferases in insects suggested that a more detailed study of the inhibition of the insect enzyme might be of value. We have therefore examined a number of compounds related to bromsulphalein in an attempt to define the structure necessary for inhibition and its relation to the active sites of an insect and a vertebrate enzyme.

* Abbreviations: DCNB, 1,2-dichloro-4-nitrobenzene; CDNB, 1-chloro-2,4-dinitrobenzene.

MATERIALS AND METHODS

Inhibitors and substrates. Phenol red (phenolsulphonphthalein), bromothymol blue (3',3''-dibromo-5',5''-diisopropyl-2',2''-dimethylphenolsulphonphthalein), chlorophenol red (3',3''-dichlorophenolsulphonphthalein), bromocresol green (3',3'',5',5''-tetrabromo-2',2''-dimethylphenolsulphonphthalein), bromophenol blue (3',5',3'',5''-tetrabromophenolsulphonphthalein), bromsulphalein (phenol-4,5,6,7-tetrabromophthalein-2',2''-disulphonic acid), eosin (2',4',5',7'-tetrabromofluorescein), 2',5'-dichlorofluorescein, 4',5'-di-iodo-1',8'-dimethylfluorescein, rose bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein), 3',5',3'',5''-tetrabromophenolphthalein and its ethyl ester were commercial samples that were used without further purification.

Succinic acid, m.p. 185°, glutaric acid, m.p. 97°, adipic acid, m.p. 150°, pimelic acid, m.p. 105°, suberic acid, m.p. 142°, azelaic acid, m.p. 106°, sebacic acid, m.p. 133°, tetradecane-1,14-dioic acid, m.p. 123°, thapsic acid (hexadecane-1,16-dioic acid), m.p. 126°, and traumatic acid (*trans*-dodec-2-ene-1,12-dioic acid), m.p. 163°, were a gift from Dr F. B. Shorland.

Recrystallized commercial samples of DCNB and CDNB were used as substrates. GSH solutions were prepared immediately before use.

Enzyme preparations. New Zealand grass grubs (*Costelytra zealandica* larvae, weight 100 mg.) were homogenized in ice-water (two grubs/ml.) in an MSE top-drive blender and the mixture was centrifuged at 15000*g* for 30 min. at 2°. The supernatant was removed and acetone at -15° added until no more precipitate formed. The precipitate was centrifuged off at -5° and the residual acetone in the deposit removed by evacuation in a desiccator, leaving a brown powder. Enzyme solutions were prepared by shaking the dry powder in water for 15 min. and centrifuging off the insoluble material at 10000*g* for 10 min. When DCNB was the substrate a 2% (w/v) suspension was made, but for CDNB assays a 0.2% suspension was sufficiently active

when 0.05 ml. of enzyme was used in a total reaction volume of 3 ml.

Sheep liver was homogenized in an equal volume of 0.1 M-pyrophosphate buffer, pH 8.3, at 0° and centrifuged at 10000g for 30 min. The supernatant was adjusted to pH 4.5 and the bulky precipitate removed by centrifugation at 10000g for 10 min. The upper layer was then restored to the pH required for subsequent work, any small precipitates being removed by centrifugation. For assays with CDNB a 1:20 dilution of this preparation was used.

Enzyme assay methods. (a) The spectrophotometric method of Booth, Boyland & Sims (1961) was used with DCNB. When the GSH concentration was varied the DCNB concentration was kept at 0.75 mM, and when DCNB concentration was varied the GSH concentration was always 2 mM.

(b) The spectrophotometric method of Cohen *et al.* (1964) with CDNB was used except that this substrate was kept at a concentration of 0.1 mM in experiments where the GSH concentration was varied. In experiments with a range of CDNB concentrations, the GSH concentration was normally kept at 4 mM. Extinction changes were followed in either the Beckman DB or the Perkin-Elmer 137 u.v. spectrophotometer and reaction rates were derived from the recorder traces. Rates of reaction were measured during the initial linear period of the reaction and individual assays were completed in less than 0.5 min. When low concentrations of substrate were used (e.g. 0.04 mM-CDNB) the assays were completed before 20% of the substrate had been converted.

Buffers were prepared from 0.1 M solutions of sodium acetate and acetic acid (pH 4.6), Na_2HPO_4 and NaH_2PO_4 (pH 6.8), or $\text{Na}_4\text{P}_2\text{O}_7$ and HCl (pH 8.10), and experiments were carried out at pH 8.3 in the pyrophosphate buffer except when a range of pH was required. All assays were carried out at 37°.

Symbols and units. The symbols used in the present work are those defined by Dixon & Webb (1964, p. 786). Reaction velocities in graphs are expressed as $\Delta E_{1\text{cm.}}/\text{min.}$

Plotting of results. The graphical methods described by Webb (1963) were used, and in experiments designed to measure the nature and constants of the inhibition five concentrations of substrate were used at each of five concentrations of inhibitor. From each set of results plots of $1/v$ against $1/s$ and $1/v$ against i were made (see Webb, 1963). From the intersection of the five straight lines on the second of these plots the experimental value of K_i was obtained and the value checked by calculation from the intercepts of the double-reciprocal plots.

Values of K_m were obtained at fixed concentrations of the second substrate, which have been quoted where appropriate. In some experiments results were plotted by each of the six graphical methods described by Webb (1963), i.e. $1/v$ against $1/s$ (type 1), $1/v$ against i (type 2), v against v/s (type 3), s/v against s (type 4), $1/(1-\alpha)$ against $1/i$ (type 5) and $i\alpha/(1-\alpha)$ against s (type 6).

RESULTS

Properties of the enzymes. Grass-grub enzyme powder was stable in a desiccator at room temperature for 2 years and activities of aqueous solutions remained constant at 5° for at least 3 weeks. Sheep-liver glutathione-S-aryltransferase activity

also remained constant at 5° for at least 3 weeks. Most grass grubs contained considerably more enzyme activity than liver. When assayed with 0.1 mM-CDNB and 5 mM-GSH at pH 8.3, an aqueous homogenate of grass grubs had an activity equivalent to 286 $\mu\text{moles/min./g.}$ of grass grub (range 37–578, 49 assays). Corresponding values for six samples of sheep-liver enzyme were 25–50 $\mu\text{moles/min./g.}$ wet wt. of liver.

Both enzymes were eluted from Sephadex G-100 columns (120 cm. $\times$ 1.5 cm.²) in 0.1 M-phosphate buffer, pH 7.4, as symmetrical peaks. Approximate molecular weights deduced from the elution volumes (Andrews, 1964) were 80 000, no significant difference being found between the enzymes from the two sources.

Effect of pH on activity. Concentrated enzyme extracts were kept for 3 min. at pH values between 5 and 10 at 37° and then diluted into the CDNB assay mixture at pH 8.3, and the reaction was started by the addition of sufficient GSH at pH 8.3 to the mixture to make a 4 mM solution. Neither sheep-liver nor grass-grub enzyme lost activity by this preliminary exposure to pH values away from the optimum.

The optimum value for both grass-grub and sheep-liver enzyme was at pH 8.3 in assays where 0.1 mM-CDNB or 0.75 mM-DCNB and 2 mM-GSH were used. With 4 mM-GSH solutions pH-activity curves of grass-grub enzyme with both aromatic substrates had a shoulder at pH 9.5–10 in addition to the peak at pH 8.3. The pH-activity curve for the non-enzymic reaction of 0.1 mM-CDNB with GSH in controls had the sigmoid form of a titration curve and the mid-point of this at pH 8.7 corresponded with the pK for the GSH thiol group given by Isherwood (1959).

Effect of pH on the inhibition. The extent of inhibition of the reaction in 0.1 mM-CDNB and 2 mM-GSH was determined at a range of pH values with bromocresol green (pK 4.9), chlorophenol red (pK 6.0), phenol red (pK 8.0) and bromosulphalein (pK 8.7). Above pH 9 the inhibition fell sharply with all the inhibitors (Fig. 1).

Nature of the inhibition. Experiments were carried out with 0.75 mM-DCNB or 0.1 mM-CDNB in which GSH concentrations ranged between 0.1 mM and 2 mM and a concentration of the inhibitors of Table 1 was chosen to give a suitable spread of values for plotting. All inhibitor experiments were plotted on graphs of types 1 and 2 and the experiments with bromocresol green on graphs of types 1–6. The forms of the graphs in each case were those of simple competitive inhibitions and the apparent values for the inhibitor constants deduced from the plots are listed in Table 1.

Some similar experiments were made in 2 mM-GSH solution with a range of aromatic substrate con-

centrations in the range 0.02–0.2mm-CDNB or 0.075–0.75mm-DCNB. Values from these experiments were also plotted on graphs of types 1 and 2, except where bromocresol green was used as inhibitor, when all six types of plot were used. In these experiments all plots had the forms of simple non-competitive inhibitions.

Fig. 1. Variation of inhibition of grass-grub glutathione S-aryltransferase with pH. Incubations were carried out with 0.1 mm-CDNB and 2 mm-GSH at 37° in the presence of: ●, 1µM-bromsulphalein; Δ, 2µM-bromocresol green; ○, 20µM-phenol red; □, 8µM-chlorophenol red.

The same values, within ±15%, were obtained for K_i values by all the plotting methods used.

Sheep-liver enzyme was not inhibited by either the dicarboxylic acids or the phthalein inhibitors at concentrations up to 0.8mm, except with 0.2mm-bromsulphalein, which caused a 15% inhibition.

Determination of K_i values for grass-grub enzyme. The method of Florini & Vestling (1957) was used (see Dixon & Webb, 1964, p. 72), assuming that the equilibria concerned are those shown:

Graphs of type 1 were constructed at pH 8.3 for five GSH concentrations between 0.04 and 0.4mm at each of five CDNB concentrations between 0.04 and 0.3mm. The intersection of the five lines gave the value of $1/K_{GSH}$ (Fig. 2). Values of $1/V_{CDNB}$ from this graph were then plotted against reciprocals of the corresponding CDNB concentrations to give a linear graph cutting the base line at the value for $-1/K'_{CDNB}$. A similar pair of graphs gave intercepts from which K_{CDNB} and K'_{GSH} were obtained.

Table 1. Apparent inhibitor constants for grass-grub glutathione S-aryltransferase at pH 8.3

Inhibitor	$10^6 K_i (M)$			
	Non-competitive		Competitive	
	CDNB (substrate) in the presence of 2 mm-GSH	DCNB (substrate) in the presence of 2 mm-GSH	GSH (substrate) in the presence of 0.1 mm-CDNB	GSH (substrate) in the presence of 0.75 mm-DCNB
Sulphonphthaleins				
Bromothymol blue	—	—	—	12.0
Bromocresol purple	—	—	—	1.75
Bromocresol green	1.75	1.1	0.15	0.75
Chlorophenol red	—	—	—	1.35
Bromophenol blue	—	—	—	0.60
Phenol red	—	—	—	20.0
Phthaleins				
Bromosulphalein	—	8.9	1.00	1.20
Tetrabromophenolphthalein	—	—	6.0	—
Tetrabromophenolphthalein ethylester	—	—	7.0	—
Fluoresceins				
Eosin	—	—	—	4.0
Rose bengal	—	—	—	5.5
Di-iododimethylfluorescein	—	—	—	7.0
Dichlorofluorescein	—	—	—	92.0
Dicarboxylic acids				
Azelaic acid	—	—	440	—
Sebaic acid	—	—	45	—
Traumatic acid	—	—	55	—
Tetradecanedioic acid	—	—	100	—
Thapsic acid	—	—	70	—

Fig. 2. Determination of K_i for GSH with grass-grub glutathione *S*-aryltransferase. GSH solutions were incubated at pH 8.3 at 37° with different concentrations of CDNB. Concentrations were: $\square$, 0.04 mM; $\blacksquare$, 0.1 mM; $\bullet$, 0.2 mM; $\circ$, 0.3 mM. Intersection is at $-1/K_i$.

The values K_{GSH} 1.7 mM, K'_{GSH} 1.54 mM, K_{CDNB} 0.87 mM and K'_{CDNB} 0.8 mM showed that the differences between K and K' for each substrate were small and that the affinity of the enzyme for each substrate was not greatly affected by its combination with the second.

Effect of pH on the enzyme and inhibitor constants. Values of K_m for GSH in 0.1 mM-CDNB solution, K_m (GSH, 0.1 mM-CDNB), and the analogous constant K_m (CDNB, 2 mM-GSH), with the corresponding values of V , were determined for grass-grub and sheep-liver enzymes between pH 5 and 10 from graphs of type 1 (Figs. 3–6). Values for v_0 for Figs. 3–6 were obtained at a concentration of 0.04 mM-CDNB (2 mM-GSH) or 0.4 mM-GSH (0.1 mM-CDNB). Values for the apparent K_i for bromocresol green were determined for the grass-grub enzyme in the presence of 0.1 mM-CDNB and 0.2–2 mM-GSH. Results of experiments were plotted on graphs of type 2 and values of K_i obtained between pH 5 and 10 are plotted in Fig. 3.

DISCUSSION

The results obtained show that inhibition of grass-grub enzyme by the phthaleins, sulphonphthaleins, dicarboxylic acids and fluoresceins was due to competition with GSH for its binding site. At a high fixed GSH concentration (2 mM) non-

Fig. 3. Combination of GSH with grass-grub glutathione *S*-aryltransferase at different pH values. Incubations were carried out at 37° in the presence of 0.1 mM-CDNB and values of v_0 , V , K_m and the apparent value of K_i for bromocresol green determined graphically as described in the text. Measurement of v_0 was at 0.4 mM-GSH.

Fig. 4. Combination of CDNB with grass-grub glutathione *S*-aryltransferase at different pH values. Incubations were carried out at 37° in the presence of 4 mM-GSH and values of v_0 , V and K_m determined graphically as described in the text. Measurements of v_0 were made at 0.04 mM-CDNB.

competitive kinetics were found when inhibitor and aromatic substrate concentrations were varied. At the concentrations used none of the inhibitors

Fig. 5. Combination of GSH with sheep-liver glutathione *S*-aryltransferase. Conditions were the same as in Fig. 3.

Fig. 6. Combination of CDNB with sheep-liver glutathione *S*-aryltransferase. Conditions were the same as in Fig. 4.

except bromsulphalein had any effect on the enzyme from sheep liver.

The phthaleins, sulphonphthaleins and fluoresceins can be regarded as isosteric with GSH, and in models it is possible to superimpose their phenolic oxygen atoms on those of the carboxyl groups of GSH. This suggested that simple dicarboxylic acids should also be tested as inhibitors. Inhibition by these acids was negligible except when the distance between carboxyl groups approached the

separation of the carboxyl groups in GSH itself. Thus sebacic acid (C_{10}) and traumatic acid (C_{12}) were the most efficient inhibitors in this group, and succinic acid, glutaric acid, adipic acid, pimelic acid and suberic acid were without action.

The inhibition did not necessarily depend on the presence of two ionized groups, such as carboxyl, at this distance since dissociation of the sulphonphthalein inhibitors did not significantly affect their inhibitory action. No obvious change of inhibition on formation of the ion occurred with phenol red (pK 8), chlorophenol red (pK 6.0) or bromocresol green (pK 4.9) (Fig. 1). Similar results were obtained with bromsulphalein (pK 8.7). The sharp fall in inhibition at about pH 9 shown in Fig. 1 appears to be related to changes in the ionization of the enzyme.

Ionization of the phthalyl carboxyl group or the corresponding sulphonic acid group in the sulphonphthaleins appears also to be irrelevant to the inhibition since tetrabromophenolphthalein ethyl ester was as effective as tetrabromophenolphthalein itself.

The pK of the inhibitor molecule is nevertheless related to the extent of inhibition found within a series of compounds of similar structure, and within each series pK values had to be less than a critical value for effective inhibition. All the sulphonphthaleins with pK values less than 6 were excellent inhibitors and those with pK values greater than 7 were much less effective (Table 1). Similar considerations seem to affect the phthaleins, where tetrabromophenolphthalein (pK 7.6) and bromsulphalein (pK 8.7) were powerful inhibitors but phthaleins with pK values greater than about 9, such as phenolphthalein or thymolphthalein, were ineffective (Cohen *et al.* 1964). Eosin (pK 1.5) was an excellent inhibitor but dichlorofluorescein (pK 5.8) was a poor one.

All the effective inhibitors among the phthaleins, sulphonphthaleins and fluoresceins were substituted with halogen atoms or sulphonic acid groups *ortho* to the phenolic hydroxyl groups; the four tetrabromo compounds, eosin, bromophenol blue and tetrabromophenolphthalein and its ethyl ester, were all about equally effective as inhibitors in spite of the wide variation in their pK values. It is possible that the two phenolic oxygen atoms in these inhibitors may simulate the effect of the carboxyl groups in GSH, and that GSH or the inhibitors are bound to the grass-grub enzyme through these groups and the two ionized centres in the active site that were detected in the kinetic studies.

The application of Dixon's (1953) rules (Dixon & Webb, 1964, p. 134) to the interpretation of the data plotted in Figs. 3–6 is complicated since the reaction is bimolecular, and pseudounimolecular conditions are only attained if the affinity of the enzyme for

each substrate remains constant when the behaviour of the second is considered.

In the alkaline range of pH, the constants for CDNB do not change (Figs. 4 and 6), so that the behaviour of GSH may be discussed with more confidence. In the sheep-liver enzyme (Fig. 5) the $\log v_0$ -pH and pK_m -pH plots for GSH indicate the presence of an ionizing group with pK 9.2 in either the enzyme or its substrate. This, from the $\log V$ -pH curve, is not present in the enzyme-substrate complex and is therefore most probably concerned in binding the GSH to the enzyme. Glutathione has a pK of 9.1 associated with the ionization of its α -NH₂ group (Isherwood, 1959), and one interpretation of the binding of GSH to the enzyme would be the pairing of this charged α -NH₃⁺ group with a negatively charged centre on the enzyme. This could be a carboxyl ionization, which would most probably have a pK below 5 and thus not appear on the pK_m or $\log v_0$ plots.

An equally probable interpretation, which seems more likely when the analogous case of the grass-grub enzyme is considered, is the matching of one of the GSH carboxylate ionic charges with a positively charged basic centre on the enzyme.

The active site of the grass-grub enzyme differs from that of the sheep-liver enzyme in that it has two ionizing centres with pK values about 9.2. These cannot be due to the GSH ionization since the slope of -2 occurs in the pK_m -pH curve as well as in the pK_m -pH and $\log v_0$ -pH curves. Plots for the grass-grub enzyme also differ from those for the sheep-liver enzyme in that a bend is present in the $\log V$ -pH plot. The pK value of the enzyme-GSH complex that is shown in this plot (Fig. 3, $\log V$ -pH) is displaced to a higher value than that in the free enzyme, and since it was not possible to measure activities satisfactorily above pH 10 it is not possible to say whether the slope on this plot should be -1 or -2 . It is also difficult to say whether the corresponding changes of slope from -2 to -1 or zero occur in the $\log v_0$ -pH and pK_m -pH curves, and these have not been shown in Fig. 3. A slope of -2 in this region would suggest that the binding between GSH and the enzyme does not mask the ionization of the enzyme, i.e. it is not due to the pairing of ionized centres as in the sheep-liver enzyme. If the slope is -1 , it would suggest that only a single ion-pair attachment of GSH is involved and that the ionization with pK 9.7 that is detected in the complex is not matched with an opposite ionic charge in the GSH. Since one ion-pair attachment can be detected in the sheep enzyme it is tempting to suggest that this is similar to that in the

grass-grub enzyme, involving a positively charged centre on the enzyme matching one of the GSH carboxyl ions. The special inhibitor-sensitivity of the grass-grub enzyme would then be due to the simultaneous presence of a second group with pK 9.2 that binds GSH through a non-ionic linkage and that does not occur in the sheep enzyme.

Possible basic groups in proteins with pK values near 9 that could occur in positively charged sites are the ϵ -NH₂ of lysine or a free α -NH₂ group. Other ionizations with pK values near 9 that might be responsible for the second, apparently non-ionic, linkage are the tyrosine OH group or a cysteine SH group.

The bends that occur at pH 6-7 in Figs. 3-6 are difficult to interpret, since changes occur with both substrates. The concentration of GSH used, 4 mM, is near to the optimum concentration at pH 8.3 and is probably sufficient to maintain near-optimum reaction rates at other pH values with low concentrations of CDNB. If this is so, both sheep-liver and grass-grub enzyme in the plots in Figs. 4 and 6 show changes that indicate an ionizing group in the CDNB binding site with a pK near 6-7. The low concentrations of CDNB used (0.1 mM) were far from saturating the enzyme, and since the constants for CDNB were changing in the region pH 6-7 the plots for GSH (Figs. 3 and 5) may be reflections of the changes in the affinity for CDNB.

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