Kinetic studies on a glutathione *S*-transferase from the larvae of *Costelytra zealandica*

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Of the glutathione *S*-transferases from the New Zealand grass grub (*Costelytra zealandica*) active in conjugating the model substrate 1-chloro-2,4-dinitrobenzene, the most active was isolated in a functionally homogeneous form. This had an isoelectric point of 8.7. Preliminary evidence suggests that it is a homodimer with subunits of M_r 23 500. The dependence of the enzyme-catalysed reaction on substrate concentration was analysed in terms of the rate equation characteristic of Ordered Bi Bi or Rapid-Equilibrium Random mechanisms. Evidence was found for a critical ionizing event at pH 9.3 at 37°C. This event appears to involve a twofold change in charge on the enzyme, which may be the result of co-operative ionizations rather than independent ionizations. This appears to affect neither the binding of the aromatic substrate to the enzyme, nor the maximum catalytic velocity of the enzyme-catalysed reaction. The variation of the kinetics with temperature was studied. Apparent thermodynamic parameters characteristic of the reaction were derived. The possible relevance of the temperature-dependence of the enzyme-catalysed reaction *in vivo* is discussed.

The glutathione *S*-transferases (EC 2.5.1.18) are a group of enzymes, found in almost all organisms studied, that have a putative detoxication function in that they catalyse the conjugation of a wide variety of electrophilic compounds with the tripeptide GSH (Boyland & Chasseaud, 1969). In insects they have been studied almost exclusively from the point of view of their role in the detoxication of many insecticides, in particular from the organophosphate class (Motoyama & Dauterman, 1980) and from the organochlorine class (Clark *et al.*, 1966; Tanaka *et al.*, 1981). The interest in this aspect of their function has increased with the observation of their involvement in the development of resistance to insecticides in houseflies (Motoyama & Dauterman, 1974, 1975, 1978; Oppenoorth *et al.*, 1979; Motoyama *et al.*, 1980) and other insects. In such cases, the resistance to such agents may be overcome if a means of blocking the detoxication reaction may be found. To this end, we have embarked on kinetic studies to explore the nature of the active site of these enzymes and the nature of the interaction of GSH with the enzyme: as GSH is the common co-substrate for a wide range of glutathione *S*-transferases, any compound that

affected the attachment of this peptide to the enzyme might be expected to be a potential synergist for a wide variety of different types of pesticide (Clark & Smith, 1975).

A preparation of glutathione *S*-transferase from the New Zealand grass grub (*Costelytra zealandica*) was one of the first to be subjected to a detailed kinetic study (Clark *et al.*, 1967). From this study it was deduced that both of the substrates employed, GSH and 1-chloro-2,4-dinitrobenzene, formed Michaelis complexes with the enzyme and that the binding of GSH to the enzyme involved the participation of two basic groups on the enzyme. The first conclusion has received implicit confirmation in subsequent kinetic studies performed on glutathione *S*-transferases isolated from other sources (Pabst *et al.*, 1974; Askelöf *et al.*, 1976; Chang *et al.*, 1981). The conclusions concerning the mode of binding of the substrate to the enzyme have not received independent confirmation, and we consider that they bear re-examination for two reasons: first, the crude, probably heterogeneous, nature of the enzyme preparation used in the original study, and, secondly, the excessively simplified experimental protocol employed, in which the concentration of only one substrate was varied at a time. In the present work a move to overcome these deficiencies has been made in that the enzyme

Abbreviation used: GSH, reduced glutathione.

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preparations used are substantially purified, being functionally homogeneous, and in that a more extended experimental procedure has been used.

In this present work we have also examined the effects of temperature variation of the kinetics of the catalytic reaction, not only as a tool to probe the active site, but also a study in its own right, to see how the detoxication process in a poikilothermic animal may be affected by environmental temperature.

Materials and methods

Chemicals

1-Chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene were obtained from BDH Chemicals, Poole, Dorset, U.K., and GSH was from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals used were of the highest grade commercially available. Wide-range (pH3–10) and narrow-range ampholytes were obtained from LKB Produkter, Bromma, Sweden.

Source of enzyme

Third-instar larvae of *Costelytra zealandica* were collected from pastures in the Canterbury and Nelson districts of New Zealand. For transport they were placed in moist soil in individual compartments of ice-cube trays. On arrival at the laboratory, they were separated from the soil and stored at -20°C until required.

Measurement of enzyme activity

During the purification procedures, enzyme activity was measured with 1-chloro-2,4-dinitrobenzene as substrate at 37°C at pH8.3, in a continuous spectrophotometric method (Cohen & Smith, 1964; Cohen *et al.*, 1964). For the kinetic studies, 1,2-dichloro-4-nitrobenzene was used as substrate, the method of Booth *et al.* (1961) being adapted for continuous assay. The reaction mixture, buffered at the desired pH, contained, in a total volume of 3 ml, GSH at one of six concentrations between 4 and 0.25 mM, and 1,2-dichloro-4-nitrobenzene at one of five concentrations between 0.6 and 0.025 mM. The reaction was initiated by the addition of 1,2-dichloro-4-nitrobenzene and was monitored at 340 nm in a Beckman Kintrac recording spectrophotometer. Reaction rates were corrected for any non-enzymic contribution. A heated cell block was used to maintain the reaction cuvettes at the required temperatures. The buffers used spanned a pH range of 8.0–10.4. Between pH8.0 and 9.9, a solution of $\text{Na}_4\text{P}_2\text{O}_7$, adjusted to the required pH with HCl, of an ionic strength of 0.755 was used. At pH10.4, 0.2 M-glycine adjusted with NaOH was used, the ionic strength being raised to 0.755 by the addition of NaCl. Buffers were adjusted to the required pH at the stipulated temperature.

Stability of the enzyme preparations

Samples of the enzyme preparation were incubated at 4°C for up to 48 h in buffers of pH between 5 and 9 and were then assayed for remaining enzyme activity. Similarly, samples of the enzyme were incubated for periods of time of up to 30 min at 37°C at pH values of between 8.0 and 9.7. Enzyme activity remaining was determined with 1-chloro-2,4-dinitrobenzene as substrate.

Preparation of enzyme

Grass grubs were homogenized in 2 vol. of 0.4 M-sodium acetate buffer, pH5.5, in an MSE top-drive blender run at full speed for 30 s, the vessel being cooled with ice. The homogenate was centrifuged at 13 000 g for 15 min. $(\text{NH}_4)_2\text{SO}_4$ was added with stirring to a final concentration of 50% saturation. The protein precipitated after 20 min of standing was removed by centrifugation at 1000 g for 10 min. The concentration of $(\text{NH}_4)_2\text{SO}_4$ in the supernatant was increased to 75% saturation. The precipitated protein was collected as described above and was dissolved in the minimum volume of 0.025 M-sodium acetate buffer, pH5.5. This solution was then dialysed, first against 5 litres of this buffer and then against 5 litres of water. Any protein precipitated by this procedure was removed by centrifugation. The remaining solution was then again fractionated by precipitation with 50%-saturated and 75%-saturated $(\text{NH}_4)_2\text{SO}_4$ as described above, except that the pH was maintained at a value of 8 by the addition of NH_3 solution during the addition of the salt. The precipitated protein was dissolved in 0.01 M-sodium acetate buffer, pH5.5, and was dialysed against two 5-litre portions of the same buffer, over 24 h. Precipitated protein was removed by centrifugation and discarded. The protein solution remaining was then purified by preparative isoelectrofocusing with wide-range (pH3–10) ampholytes. Fractions corresponding to the two main enzyme peaks, at pI8.7 and 4.6, were pooled and dialysed against 0.025 M-sodium acetate buffer, pH5.5. The pI8.7 enzyme was stored in 2 ml portions at -20°C until required.

Preparative isoelectrofocusing

This procedure was carried out at $5-6^{\circ}\text{C}$ in a vertical apparatus similar to the LKB 8100. The pH gradient was established with a 1% ampholyte solution and was stabilized with a stepwise sucrose gradient. The anode was at the bottom of the apparatus and power consumption was limited to 0.7 W. The runs lasted for 80–140 h, the focusing pattern being stable during this period.

Protein determination

Protein concentration was monitored spectrophotometrically by the procedure of Layne (1957)

or was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Analysis of kinetic data

Initial-velocity measurements and also derived parameters were fitted to the appropriate equations by non-linear-regression techniques as described by Wilkinson (1961). Crude rate data were fitted to the rate equation used by a modification of the program published by Cleland (1967), incorporating a routine for the automatic revision of weighting factors during each cycle of iteration. Derived parameters were fitted to the appropriate equation and were weighted according to the variance calculated for them during the initial regression.

Results

Enzyme purification

Costelytra zealandica is known to possess a number of glutathione *S*-transferases. The most prominent (about 75% of total activity) with regard to its activity with 1-chloro-2,4-dinitrobenzene is that isoelectric at pH8.7–8.8 (Clark *et al.*, 1973). The purpose of the enzyme purification was to obtain this enzyme in a functionally homogeneous state. On the basis of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli & Favre, 1973) the purity of the protein was estimated to be 70–75%, there being one major contaminant of large molecular mass. In the *M_r* range 20 000–25 000 there appeared to be only a single protein band, the major protein component, with a mobility corresponding to *M_r* 23 500 ± 500. No other protein bands within this *M_r* range were seen, and it is thus concluded that the enzyme obtained was a homodimer and further that the preparation was uncontaminated by other glutathione *S*-transferases of different subunit constitution (Bass *et al.*, 1977; Chang *et al.*, 1981). Narrow-range isoelectrofocusing of the purified pI8.7 enzyme revealed the presence of only one zone of enzyme activity.

The pI4.6 enzyme also possessed substantial activity with 1-chloro-2,4-dinitrobenzene. It was not studied in the present work, since sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and iso-

Fig. 1. Variation of kinetics of the *Costelytra* glutathione *S*-transferase with substrate concentrations
In (a) GSH concentrations were held constant at: ○, 4 mM; ●, 3 mM; △, 1 mM; ▲, 0.5 mM; □, 0.25 mM. In (b) constant 1,2-dichloro-4-nitrobenzene concentrations were: ○, 0.75 mM; ●, 0.45 mM; △, 0.15 mM; ▲, 0.075 mM; □, 0.025 mM. The experimental points shown in (a) and (b) are both taken from the same data set, but for the sake of clarity some sets of points have been omitted in both diagrams. The continuous lines indicate computed lines of best fit. The experiment was performed at pH 8.7 at 37°C.

Table 1. Purification of glutathione *S*-transferases from *Costelytra zealandica*

	Volume (ml)	Enzyme units (μmol/min)	Protein (mg/ml)	Specific activity (μmol/min per mg)	Yield (%)
13 000 g supernatant	4700	272 000	29	2.0	100
50–75%-satn.-(NH ₄) ₂ SO ₄ fraction (pH 5)	250	71 500	19.5	14.7	26
50–75%-satn.-(NH ₄) ₂ SO ₄ fraction (pH 8)	94	38 000	13.5	30	14
Isoelectrofocusing (pH 3–10): pI 8.7 fraction	101	5500	0.38	143	2.3
Isoelectrofocusing (pH 4–6): pI 4.6 fraction	200	3180	1.8	8.8	1.2

electrofocusing indicated an unacceptable degree of heterogeneity. This preparation had a very low specific activity compared with the pI8.7 enzyme. However, preliminary studies on this preparation have shown that its responses to changes in the concentrations of the substrates and to changes in pH are almost identical with those of the pI8.7 enzyme.

The course of the preparation is shown in Table 1. Work carried out subsequently to that discussed here has shown that this enzyme may be prepared in higher yield (about 15%) by use of the affinity-chromatography technique employed by Chang *et al.* (1981). The various isoenzymes found in grass-grub preparations differ quite markedly in their behaviour on this medium (A. G. Clark, G. L. Dick, S. M. Spier & J. N. Smith, unpublished work).

Stability of the enzyme preparations

The enzyme preparation used for kinetic studies was tested for stability. It was found that, under the assay conditions used, losses of activity during the assay could be kept to less than 5% at the most extreme conditions.

Kinetic studies

The variation of the rate of the enzyme-catalysed reaction was studied over a range of concentrations of both substrates and at different pH values, as described in the Materials and methods section. Results of a typical experiment are shown in Fig. 1. The experimental points displayed in $[S]/v$ -versus- $[S]$ plots (Hanes, 1932) approximate closely to a pencil of straight lines intersecting to the left of the vertical axis when either substrate was varied. These results were therefore fitted to the equation:

$$v = \frac{V}{1 + \frac{K_a}{[A]} + \frac{K_b}{[B]} + \frac{K_{ia}K_b}{[A][B]}} \quad (1)$$

where $[A]$ and $[B]$ represent the concentrations of GSH and 1,2-dichloro-4-nitrobenzene respectively, V is the maximum velocity, K_a and K_b are the Michaelis constants with respect to GSH and 1,2-dichloro-4-nitrobenzene respectively and K_{ia} is an 'inhibition constant' with respect to GSH as defined by Cleland (1963). This model gave acceptable values for V , K_{ia} and K_b , coefficients of variation being of the order of 5–15%. The values of K_a obtained with this regression procedure were in general much less satisfactory, the coefficients of variation ranging from 10 to 150% with a median value of approx. 40%.

The way in which the regression parameters V , K_a , K_{ia} and K_b varied with pH was examined. Results obtained are shown in Fig. 2; V and K_b were, within experimental error, invariant with pH.

Fig. 2. Variation of kinetic parameters of a glutathione *S*-transferase from *Costelytra zealandica* with pH. The parameters V , K_{ia} , K_b and K_a were determined at 37°C as described in the text, and were plotted logarithmically against pH. Broken lines show computed lines of best fit.

The weighted means for these two parameters were calculated. The value of $\bar{V}$ obtained was 13.8 nmol/min. The value of $\bar{K}_b$ obtained was 1.13 ± 0.2 mM.

The value of K_{ia} increased with increasing pH, the final portion of the log-log curves having a gradient approximating to a value of -2 . The variation of K_{ia} with pH was analysed according to three possible models. The first assumed that the catalytically active form of the enzyme was protonated and that the change in K_{ia} values was due to a single deprotonation step. The equation predicted from this model is:

$$K_{ia} = \bar{K}_{ia} / (1 + K_1/[H^+]) \quad (2)$$

where K_{ia} is the value determined experimentally at the various pH values, and $\bar{K}_{ia}$ is the pH-independent constant characterizing the catalytically active form of the enzyme (Dixon & Webb, 1964, pp. 128–144).

The second model requires a doubly protonated active species that ionizes in successive stages, through inactive singly ionized species, to a double anion. The equation generated by this model is:

$$K_{ia} = \bar{K}_{ia} / (1 + K_1/[H^+] + K_1K_2/[H^+]^2) \quad (3)$$

Table 2. Variation of kinetic parameters of *Costelytra* glutathione *S*-transferase (pI8.7) with temperature
Abbreviation: DCNB, 1,2-dichloro-4-nitrobenzene.

Temperature (°C)	V (nmol/min)	K_{ia} (mM)	K_b (mM)	pK (M)
15	2.40 ± 0.55	0.046 ± 0.013	0.767 ± 0.23	9.77 ± 0.1
22	4.75 ± 0.33	0.089 ± 0.01	0.790 ± 0.09	9.93 ± 0.1
30	9.6 ± 2.0	0.177 ± 0.03	1.13 ± 0.25	9.59 ± 0.06
37	13.8 ± 2.1	0.257 ± 0.04	1.13 ± 0.21	9.36 ± 0.12
Apparent thermodynamic parameters				
Parameter	Value (kJ/mol)	Parameter	Value (kJ/mol)	
ΔH^0 (DCNB binding)	17.6 ± 5	ΔH^0 (GSH binding)	56 ± 4	
ΔG^0 (DCNB binding)	17.5 ± 0.5	ΔG^0 (GSH binding)	22.0 ± 0.1	
$\Delta H^\ddagger$ (Catalytic reaction) = 54 ± 4 kJ/mol.				

where K_{ia} and $\tilde{K}_{ia}$ are as above and K_1 and K_2 are the molecular acid dissociation constants. The same equation applies even when one ionizing pathway is completely dominant.

The third model involves a twofold ionization in which the release of two protons is a concerted event. The equation describing the type of behaviour predicted by this model is:

$$K_{ia} = \tilde{K}_{ia} / (1 + K^2 / [H^+]^2) \quad (4)$$

where K^2 is a third-order dissociation constant, K being the concentration of H^+ giving 50% of the maximal catalytic activity.

Of these models, the first two were found to fit the experimental results poorly; in particular, the second required negative values for K_1 and K_2 . The third equation yielded a good fit to the experimental findings, and the value of K obtained was 9.36 ± 0.07 (S.E.M.). The value of $\tilde{K}_{ia}$ obtained was 0.257 ± 0.025 mM. An attempt was made to refine the regression parameters by an iterative procedure analogous to that described by Wilkinson (1961), but this produced no improvement in the fit. The values of K_a at the various pH values were characterized by large coefficients of variation, and it was not possible to discern any consistent trend in the variation of this parameter with pH. No attempt was made to fit these values of any of the above models.

The experiment as described above was repeated at 15, 22 and 30°C with the pI8.7 preparation. Decreasing the temperature did not generate any qualitative change in the form of the results. As the temperature decreased the errors on the regression parameters increased, but it was possible to obtain good estimates of V , $\tilde{K}_{ia}$ and $\tilde{K}_b$ at these temperatures. These values are shown in Table 2. From these were obtained notional thermodynamic parameters. From the variation of V with temperature an apparent enthalpy of activation ($\Delta H^\ddagger$) for the catalytic reaction was obtained. From the

variation of K_{ia} and K_b values the apparent enthalpies of binding of glutathione and 1,2-dichloro-4-nitrobenzene were obtained (Dixon & Webb, 1964, pp. 145–146) and from the values of these parameters at 37°C, the apparent change in standard free energy on the binding of the substrates was calculated. These quantities are also shown in Table 2.

Effect of temperature on the spontaneous reaction

The uncatalysed reaction with 1,2-dichloro-4-nitrobenzene was generally so slow that it was not possible to measure the rates with acceptable accuracy. However, to give an idea of the effect of the enzyme on the conjugation reaction, the spontaneous and enzyme-catalysed reaction were studied with 1-chloro-2,4-dinitrobenzene as substrate. The reactions were performed at pH 8.3, with 0.1 mM 1-chloro-2,4-dinitrobenzene and 2 mM-GSH at up to ten temperatures between 15 and 40°C. In both instances the Arrhenius plots derived from the rate measurements were linear. The gradients corresponded to apparent activation energies of 64 ± 2 and 29 ± 3 kJ/mol for the spontaneous and enzyme-catalysed reactions respectively.

Discussion

The findings reported here confirm the results presented in earlier work and remove some of the ambiguity associated with the earlier paper (Clark *et al.*, 1967). In the previous studies the probable heterogeneity of the preparation rendered it difficult to ascribe the kinetic characteristics observed to any single enzyme species. The pI8.7 preparation used in the present work was found to be catalytically homogeneous, so that changes observed could be attributed to one enzyme species. Secondly, the experimental format employed in the present work, in which the concentrations of both substrates were varied in the course of a single experiment, means that the kinetic parameters determined are largely

independent of substrate concentrations. We confirm observations made in the earlier paper: the maximum velocity of the reaction and the apparent dissociation constant for the enzyme–1,2-dichloro-4-nitrobenzene complex are independent of pH between pH 8 and 10, whereas the value of K_{ia} , which may in the first instance be interpreted as the dissociation constant of the enzyme–GSH complex (Cleland, 1963), does vary with pH in this region. A fitting of the values of K_{ia} obtained to three possible models suggests that this parameter appears most nearly to vary inversely with the square of the H^+ concentration. This, too, conforms with the earlier observations.

However, an examination of the way in which the experimental findings fit the equation indicates that K_a is a rather poorly defined parameter in almost all the experiments. This unequal distribution of errors suggests that eqn. (3) may not be the most appropriate to describe the behaviour of the enzyme. Graphical presentation of the results reveals a tendency for many of the velocity measurements made at the lowest GSH concentration to deviate from the regression lines, the velocities being lower than predicted by the equation. This tendency is apparent, but not marked, in Fig. 1(b). Because of the limited range of concentration employed in the present work, it was not feasible to fit to an equation involving higher-order terms in GSH concentration, but it seems very likely that the behaviour of the enzyme examined here is similar to that reported for another insect glutathione *S*-transferase, that from the larvae of *Galleria mellonella*, in which the behaviour of the enzyme with respect to the electrophilic substrate was Michaelian but was non-Michaelian with respect to GSH. The mechanism invoked in this instance was Steady-State Random (Chang *et al.*, 1981; Askelöf *et al.*, 1976). It seems probable that the large errors in the determination of K_a are due to the absence from the rate equation of higher-order terms involving only the concentration of GSH: for the purpose of the present analysis it is assumed that the numerical values of V , K_{ia} and K_b would not be substantially affected by the inclusion of such terms. The low value of the coefficients of variation obtained with these latter parameters supports this assumption.

The change in the value of K_{ia} with pH has been attributed to an ionizing event involving the free enzyme only (Clark *et al.*, 1967). The most plausible explanation of the behaviour observed is that a co-operative dissociation of H^+ is involved: that the dissociation of a basic group (that governed by K^2) results in a conformational change, which in turn results in the immediate dissociation of a previously shielded group. Co-operative ionization in a slightly different model has been proposed by other workers (Brocklehurst & Dixon, 1976). The data acquired in

the present study are not compatible with the simultaneous ionization of two independent groups: such a mechanism would require that eqn. (2) should apply, the values of K_1 and K_2 being equal.

The possibility of a co-operative ionization occurring suggests that an earlier interpretation of these change in behaviour in the region of pH 9 should be questioned. In an earlier publication (Clark *et al.*, 1967) it was proposed that GSH is bound to the enzyme through two basic residues interacting with the carboxylic acid residues on GSH. However, if one of the ionizations observed is due to the unshielding of a group normally buried in the interior of the enzyme, then it seems unlikely that this group would interact with GSH. In the same paper, it was observed that this behaviour did not appear to occur in an enzyme preparation from sheep liver, a change in gradient of only -1 rather than -2 being found in this region. In the light of the above discussion, if the two ionizations proposed for the *Costelytra* enzyme do not both directly affect the binding of GSH, then the differences between the two enzymes may not reflect a fundamental difference in catalytic mechanism. This would be compatible with other findings from this laboratory that support the idea of their being very strong similarities between the glutathione *S*-transferases of insect origin and those from vertebrate sources (Chang *et al.*, 1981; A. G. Clark & B. Drake, unpublished work).

In sum, these findings indicate that the possibility of preferentially blocking the conjugation with GSH of pesticides in insects, exploiting differences between the insect and vertebrate enzymes (Clark *et al.*, 1967; Clark & Smith, 1975), now seems a less promising prospect.

The ways in which the net catalytic rates and the derived parameters varied with temperature was studied. Preliminary studies with the more reactive substrate 1-chloro-2,4-dinitrobenzene showed that the addition of the enzyme did, as is expected in catalysis, lead to a decrease in the apparent activation energy for the reaction, in this instance by a factor of 2. More detailed studies were made with 1,2-dichloro-4-nitrobenzene as the substrate. The mean values of the maximum velocity varied over a 5-fold range (see Table 2). Assuming that the maximum velocity represents the product of a pseudo-first-order rate constant and the enzyme concentration, the dependence of this parameter on temperature corresponds to an enthalpy of activation of 54 kJ/mol (E_a 57 kJ/mol). This is rather higher than the corresponding values quoted by Motoyama & Dauterman (1977) for partially purified glutathione *S*-transferases from two strains of housefly. The differences may merely represent differences in experimental protocol: in the work cited above the velocities measured were not maximum velocities and the measurements were

made at a single pH, removed from the apparent pH optimum.

The values of K_{ia} and K_b were also found to vary with temperature. K_b increased by 50%, but in contrast K_{ia} increased by about 500%, over the temperature range examined. On the assumption that these derived parameters are in fact dissociation constants for the respective enzyme-substrate complexes, then this behaviour may be summarized in thermodynamic terms as follows. The apparent free energies of binding of the two substrates at 37°C are rather similar (Table 2). The enthalpies of binding are quite different, and this difference may be explained by invoking very different entropy changes on the binding of each substrate: 0 ± 20 and 110 ± 15 kJ/mol per °C for 1,2-dichloro-4-nitrobenzene and GSH respectively. This observation clearly indicates different modes of binding for the two substrates, but it would be premature to attempt a detailed mechanistic explanation.

Although a detailed explanation of the behaviour of the enzyme is not possible at present, the data do describe unambiguously the way in which the enzyme responds to temperature, and this is of particular interest in view of what may be required of a detoxication enzyme in a poikilothermic organism. The parameter K_b in eqn. (1) describes the response of the enzyme to variation of 1,2-dichloro-4-nitrobenzene, and the parameter K_{ia} together with K_a describes the response to variation in the concentration of GSH. The value of K_{ia} varies over a 5-fold range over the temperature range examined; this is likely to be of little functional significance given the values of K_{ia} computed and the probable concentrations of GSH in the tissues (3–10 mM: Griffith & Meister, 1979; Saleh *et al.*, 1978). The enzyme is likely to be fully saturated with GSH at all of these temperatures. It is, in fact, the near constancy of K_b that may be of significance. A toxic nucleophile such as 1,2-dichloro-4-nitrobenzene would be expected to be in the cell at a relatively low concentration, far from saturating the enzyme. As the temperature rose it would be expected to react with cellular nucleophiles at an enhanced rate. Enzyme-catalysed conjugation would also increase. The parameter governing the detoxication of this compound at low concentration is not V but the pseudo-first-order constant V/K_b . If K_b increases its value with temperature in parallel with V , then the value of the effective rate-determining constant will remain nearly constant. The rate of detoxication will lag behind that of intoxication. On the other hand, if the value of K_b is constant, the value of the pseudo-first-order parameter will rise in parallel with that of V : detoxication will keep pace with or even overtake the process of intoxication. The difference in behaviour discussed is illustrated in the two sets of data available in the present work. From Fig. 3 it is

Fig. 3. Variation with temperature of the kinetic parameters of a glutathione *S*-transferase from *Costelytra zealandica*

Parameter values are plotted as follows: Δ , $V \times 10^9$ (mol/min); $\bullet$, $V/K_{ia} \times 10^5$ (litre.min⁻¹); $\circ$, $V/K_b \times 10^6$ (litre.min⁻¹). Error bars indicate the standard deviations.

clear that V/K_{ia} remains almost exactly constant over the whole of the temperature range examined. If availability of GSH were the limiting factor in the detoxication of 1,2-dichloro-4-nitrobenzene *in vivo*, then the rate of detoxication would remain constant as the temperature rose, whereas the rate at which this compound modified cellular constituents would increase rapidly. On the other hand, the value of V/K_b increases with increased temperature and thus, in the situation where the toxicant is present in limiting concentrations, detoxication will tend to keep pace with enhanced rates of intoxication.

It has been well known for many years (see, e.g., Lindquist *et al.*, 1945; Guthrie, 1950; Young & Roussel, 1958; Hassan *et al.*, 1970) that the toxicity of insecticides varies with temperature in a fashion dictated by the class of insecticide, the species and even strain of insect and the stage of its life cycle. These effects are the end result of a multitude of interacting factors. Penetration rates have been

observed to increase with temperature, but there is no direct evidence that enhanced metabolism is responsible for negative toxicity-temperature trends. The rather striking thermodynamic characteristics of the enzyme studied in the present work suggest, however, that changes in rates of metabolism relative to rates of toxic action could be important factors in determining the effects of temperature change on the action of some insecticides.

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