Purification and properties of glutathione *S*-transferases from larvae of *Wiseana cervinata*

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1. The glutathione *S*-transferases from the porina moth, *Wiseana cervinata*, were purified by affinity chromatography, cation-exchange chromatography and preparative isoelectrofocusing. The major transferase (IV) was purified to homogeneity by a factor of 530-fold with a yield of 83%. Other transferases present were purified to a smaller degree (approx. 50-fold) to a stage of near-homogeneity. 2. The transferases examined all had M_r values about 45 000–50 000. They appeared to be homodimers of either of two types of subunit, of M_r 22 800 and 24 600. Enzymes consisting of the different types of subunit were not immunologically cross-reactive. 3. The major enzyme fractions separated by cation-exchange chromatography were both active with 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, ethacrynic acid and iodomethane, but were inactive with 4-nitropyridine *N*-oxide, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, bromosulphophthalein and *p*-nitrobenzyl chloride. 4. The kinetics of the enzyme-catalysed reaction with enzyme IV were non-Michaelian with respect to both substrates. Both products were inhibitory. The results appear to be compatible with a random steady-state mechanism. 5. It is concluded that these enzymes are very similar, in their physical and chemical constitution, in their catalytic properties and in their relationships with each other, to those enzymes that have been isolated from vertebrate organisms.

The glutathione *S*-transferases (EC 2.5.1.18) are a group of detoxification enzymes that act by catalysing the conjugation of compounds that are toxic, by virtue of their possession of an electrophilic reactive centre, with the nucleophilic thiol group of GSH. They are extremely widespread in Nature (for a review see Jakoby, 1978), but detailed studies on the molecular properties of these enzymes have to date been confined almost entirely to those isolated from mammals. Observations that resistance to insecticides in insects may be attributable to enhanced GSH-dependent detoxification (Oppenorth *et al.*, 1979; Motoyama *et al.*, 1980) has focused interest on these enzymes in insect pest strains. In the current study, the aim has been to obtain information that will enable a detailed comparison of the molecular properties of the enzymes from an insect (an economically significant pasture pest in New Zealand) with those of the mammalian enzymes that have been studied in depth. Such comparisons are necessary if it is ever to be possible to exploit species differences in the nature of GSH-dependent detoxifications in the production of pesticides of high species-specificity.

Abbreviation used: GSH, reduced glutathione.

Materials and methods

Materials

Substrates for enzyme assays were 1,2-dichloro-4-nitrobenzene (Eastman-Kodak, Rochester, NY, U.S.A.), 1-chloro-2,4-dinitrobenzene (BDH Chemicals, Poole, Dorset, U.K.), 1-menaphthyl sulphate and bromosulphophthalein (sodium salt) (both from Sigma Chemical Co., St. Louis, MO, U.S.A.). Ethacrynic acid [2,3-dichloro-4-(2-methylenebutyl)phenoxyacetic acid] was a gift from Merck, Sharpe and Dohme, Rahway, NJ, U.S.A. L-Glutathione (GSH) was purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Methanesulphonic acid (4M), containing 0.2% 3-(2-aminoethyl)indole, was from Pierce Chemical Co., Rockford, IL, U.S.A. DEAE-cellulose (DE-32) was from Whatman, Maidstone, Kent, U.K. CM-Sepharose, Sepharose 4B and Pharmalyte ampholytes were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Freund's complete and incomplete adjuvants were obtained from Difco Laboratories, Detroit, MI, U.S.A.

The conjugate of GSH and bromosulphophthalein was prepared and the immobilization of this conjugate on CNBr-activated Sepharose 4B was

performed as described previously (Clark *et al.*, 1977; Clark & Dauterman, 1982). *S*-(2,4-Dinitrophenyl)glutathione was prepared as described by Hollingworth *et al.* (1973).

Enzyme assays

Enzyme activity was monitored by measuring the rate of conjugation of 1-chloro-2,4-dinitrobenzene with GSH at 30°C and pH 6.5. This and the assays with the other substrates used were performed as described by Habig *et al.* (1974). Enzyme activity is expressed throughout as $\mu\text{mol/min}$ and specific activity as $\mu\text{mol/min per mg}$ of protein.

Enzyme source

Larvae of *Wiseana cervinata* (1g approx.) were collected from infested soil in the Wellington region during the period May–October. They were stored in batches of 100–200g at -20°C until required.

Protein concentration

This was monitored during chromatographic procedures by measurement of the absorbance at 280nm. For the determination of specific activities protein concentrations were determined either by the method of Lowry *et al.* (1951) or by the dye-binding method of Bradford (1976). Bovine serum albumin was used as a standard.

Electrophoretic analyses

Proteins were examined at all stages by disc electrophoresis in polyacrylamide gel (Davis, 1964; Ornstein, 1964), by electrophoresis in sodium dodecyl sulphate in continuous (Weber & Osborn, 1969) and discontinuous (Laemmli, 1970) buffer systems, and by isoelectrofocusing in polyacrylamide gel (Wrigley, 1971). Protein zones were detected by staining with Coomassie Blue R. Stained gels were scanned at 620nm with an ISCO model 310 gel scanner. Enzyme activity with respect to methyl iodide was localized by the method of Clark (1982).

Preparation of antibodies

Antiserum to the protein to be studied was prepared by inoculating male New Zealand White rabbits by standard methods. A γ -globulin fraction was prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation and ion-exchange chromatography (Herbert *et al.*, 1973). This fractionated antiserum was used in subsequent experiments.

Double immunodiffusion was performed in 2mm layers of 1% agarose as described by Fleischner *et al.* (1972). Immunoelectrophoresis in 2mm layers of agarose was as performed by Ouchterlony (1958). Immunotitrations were performed by the method of Hales & Neims (1976), with poly(ethylene glycol) as a precipitating agent.

Enzyme preparation

Batches of 150g of *Wiseana* larvae were homogenized for 1min in 400ml of 20mM-sodium phosphate buffer, pH 7.4 (buffer A). The homogenate was strained through cheesecloth and was centrifuged at 18000g for 30min at 4°C . The supernatant was subjected to combined anion-exchange chromatography and affinity chromatography on GSH–bromosulphophthalein conjugate immobilized on Sepharose 4B, essentially as described by Clark & Dauterman (1982). All fractions containing significant enzyme activity with respect to 1-chloro-2,4-dinitrobenzene were pooled. GSH and glycerol were added as stabilizers to final concentrations of 5mM and 25% (v/v) respectively. The pooled material was concentrated by vacuum ultrafiltration through dialysis tubing to approx. 6ml and was then stored at -20°C .

Concentrated samples from this step were adjusted to pH 6.5 with 10mM- NaH_2PO_4 solution and were then applied to a column (2.6cm $\times$ 40cm) packed with CM-Sepharose that had previously been equilibrated with 10mM-sodium phosphate buffer, containing 25% (v/v) glycerol and 5mM-GSH, pH 6.5 (buffer B). The column was developed in this buffer until the absorbance of the eluate at 280nm had fallen to a value of less than 0.01. A gradient (10–350mM; 460ml) of sodium phosphate buffer, pH 6.5, containing the same stabilizers as above was then applied to the column. Fractions of volume 8ml were collected. Conductivity, absorbance at 280nm and enzyme activity were determined for each fraction of eluate. The active fractions of each peak were pooled separately, concentrated and stored at -20°C .

Relative molecular mass and subunit composition

The M_r of purified enzymes was determined by using gel-permeation chromatography on columns of either Sephadex G-75 (2.4cm $\times$ 40cm) or Sephacryl S-200 (1.6cm $\times$ 100cm). The columns were calibrated and M_r values determined by the method of Andrews (1964). Standard proteins used for the calibration of the columns were lysozyme, myoglobin, chymotrypsinogen A, pepsin, ovalbumin and bovine serum albumin. Subunit composition was examined by sodium dodecyl sulphate electrophoresis. The M_r standards employed were a series of cross-linked oligomers of lysozyme prepared as described by Payne (1973).

Amino acid composition

The glutathione *S*-transferase from peak IV was chromatographed on a column (1.6cm $\times$ 100cm) of Sephacryl S-200 to remove GSH and glycerol. The active fractions were collected, concentrated to 5ml and then dialysed for 72h against two changes of

2 litres of distilled water. Duplicate samples were hydrolysed for 12, 24 and 72 h by the method of Hirs (1967). Amino acid contents were determined by Dr. P. Davis of the Wellington Cancer and Medical Research Institute. Tryptophan was determined after hydrolysis in 4 M-methanesulphonic acid (Simpson *et al.*, 1976).

Enzyme kinetic studies

The rate of conjugation of 1-chloro-2,4-dinitrobenzene with GSH was monitored spectrophotometrically at 340 nm, with a Varian Cary 210 double-beam spectrophotometer, at a constant temperature of 30°C. Each substrate was varied over about 100-fold range of concentration at several fixed concentrations of the other. The range used for 1-chloro-2,4-dinitrobenzene was 0.02–1 mM and that for GSH was 0.02–5 mM. The procedures adopted for the study of the effects of the variation of substrate concentration and of the product inhibitors *S*-(2,4-dinitrophenyl)-L-glutathione and NaCl were those employed in a previous study (Chang *et al.*, 1981).

Optimum pH

The activity of the enzyme preparations was examined at pH values ranging from pH 6.2 to 10.7. The concentrations of 1-chloro-2,4-dinitrobenzene and GSH were both held at 1 mM and the temperature was 30°C. Buffers used were 0.1 M-sodium phosphate (pH 6.2–8.0), 0.05 M-Tris/HCl (7.4–9.0), 0.05 M-glycine/NaOH (pH 9.0–10.4) and 0.1 M-sodium carbonate (pH 10.0–10.7). The reaction was started by adding 5 μ l of enzyme solution to the mixture.

Results

Enzyme purification

The first step, combining anion-exchange and affinity chromatography, resulted in a substantial increase in specific activity (100-fold) of the enzyme preparation. On cation-exchange chromatography (Fig. 1) the presence of four peaks of protein was revealed, three of which were associated with enzyme activity with 1-chloro-2,4-dinitrobenzene as substrate. By far the greatest activity was found in the peak retained most strongly on the column (IV). The next most active peak was that which emerged in the void volume. A trace of activity was consistently found with peak III, which contained a green pigment. Activity with 1,2-dichloro-4-nitrobenzene as substrate was found in peaks I and IV, but was not detected in peak III. The course of the purification to this point is shown in Table 1.

On preparative isoelectrofocusing a further apparent multiplicity of these enzymes was found. Peak

IV consisted largely of a protein isoelectric at pH 8.5 (IVb) but contained a variable amount (5–15%) of enzyme isoelectric at pH 9.0 (IVa). Peak I was shown to comprise two catalytic components isoelectric at pH 8.3 (Ia) and 7.5 (Ib). The peak-III material was characterized by enzymically active bands focusing at pH 8.0 and pH 8.6. It seems likely that the higher-pI component was a contaminant from peak IV (see Fig. 2). In the remainder of this paper the various enzyme preparations are described as I, III, IVa and IVb etc. as described above.

There is, as yet, no information as to whether the various enzyme forms are characteristic of different tissues. Experiments with single larvae indicate a similar multiplicity of enzyme forms in individuals, thus suggesting that the results above are not merely the result of population heterogeneity in the samples used.

Homogeneity of enzyme preparations

The enzyme designated IVb was found to be homogeneous by three criteria: by polyacrylamide-gel electrophoresis (Davis, 1964), by sodium dodecyl sulphate / polyacrylamide - gel electrophoresis (Laemmli, 1970) and by analytical isoelectrofocusing in polyacrylamide gel. Furthermore the enzyme IVa was found to behave identically with enzyme IVb on analytical gel electrophoresis in both the presence and the absence of sodium dodecyl sulphate. It seems likely that enzyme IVa may be an artifact of the preparative isoelectric focusing.

The heterogeneity in peak I appeared to be genuine in that the proteins Ia and Ib consistently migrated at different rates on analytical electrophoresis (Fig. 3). That both enzyme Ia and enzyme Ib had catalytic activity with methyl iodide, as did enzyme IVb, is shown in the zymogram in Fig. 5. The enzyme Ib was found to be heterogeneous by analytical electrophoresis and isoelectrofocusing. One catalytically active band was found in the isoelectrofocusing gels with an isoelectric point of approx. 6.5 as well as several catalytically inactive proteins. Enzyme Ia appeared to be homogeneous on analytical electrophoresis, but on analytical isoelectrofocusing it became apparent that it was contaminated by traces of enzyme Ib. The major catalytically active protein in the fraction Ia had an isoelectric point of 7.38, as measured after gel isoelectrofocusing.

Relative molecular mass and subunit studies

The M_r values of the enzymically active proteins in fractions I and IV were determined by chromatography on a column of Sephacryl S-200. The peak of enzyme activity eluted in almost exactly the same volume with both preparations, corresponding to M_r 39 700 $\pm$ 700 (mean $\pm$ s.d.).

The subunit structures of proteins in fractions IV,

Fig. 1. Separation by cation-exchange chromatography of glutathione *S*-transferases from larvae of *W. cervinata*. A partially purified preparation of the enzymes, obtained by affinity chromatography of a crude homogenate, was chromatographed on CM-Sepharose at pH 6.5 as described in the Materials and methods section. Activity was determined with 1-chloro-2,4-dinitrobenzene (○) and 1,2-dichloro-4-nitrobenzene (●) as substrates. Activity with 1,2-dichloro-4-nitrobenzene as substrate is obtained by multiplying the given scale by 0.025. ----, A_{280} .

Table 1. Purification of glutathione *S*-transferases from *Wiseana cervinata*
For experimental details see the text.

Procedure	Total protein (mg)	Total activity ($\mu\text{mol/min}$)	Specific activity ($\mu\text{mol/min per mg}$)	Purification (fold)	Yield (%)
Homogenate	32090	6489	0.2	1	100
Affinity chromatography	340	7058	20.7	103	109
CM-Sepharose chromatography					
I	73	650	9.0	45	10
II	34	320	0.95	4.7	0.5
III	70	240	3.5	17.3	4.0
IV	49.5	5360	108	540	83

IVa, IVb and I were examined by the high-resolution sodium dodecyl sulphate/polyacrylamide-gel electrophoretic system described by Laemmli (1970). Fraction IV was seen to possess traces of large-molecular-mass components, but all the other samples appeared to contain only a single protein species. The proteins in fractions IVa and IVb appeared to have identical M_r values of 22800 ± 200 , whereas the single species found in the fraction I tracks had an M_r determined to be 24600 ± 300 (Fig. 4). All enzymes species studied thus appear to be homodimers.

Immunological relationships

An antiserum to fraction IVb was prepared as

described in the Materials and methods section. This antiserum was effective at precipitating fraction IVb, in the immunotitration procedure described in the Materials and methods section. The antiserum possessed no ability to precipitate the enzymes in fraction I. There was no detectable cross-reaction with glutathione *S*-transferases purified from rat liver, sheep liver or adult housefly, or from larvae of another insect, *Galleria mellonella*. Fraction III was also found to cross-react, and it was possible to obtain almost quantitative precipitation of the activity in this enzyme preparation.

Ouchterlony immunodiffusion and immunoelectrophoresis gave qualitative confirmation of the above results. The enzymically inactive protein in

Fig. 2. Preparative isoelectrofocusing of glutathione *S*-transferases from *W. cervinata*

The active fractions designated I, III and IV in Fig. 1 were subjected to further fractionation by isoelectrofocusing in wide-range (3.5–10, III) and narrow-range (8.5–10, I and IV) pH gradients. Activities were determined with 1-chloro-2,4-dinitrobenzene as substrate.

fraction II gave no immunoreaction with the antiserum.

Substrate specificity

As shown in Table 1, the enzymes from fraction IV had a high specific activity with 1-chloro-2,4-dinitrobenzene, and the preparation of fraction I had a moderate activity (108 and 9 μmol/min per mg of protein). The enzymes had similar but not

Fig. 3. Analytical electrophoresis of glutathione *S*-transferases from *W. cervinata*

Electrophoresis was performed in polyacrylamide gel slabs. Enzyme samples were as follows: (a) fraction IVa; (b) fraction Ia; (c) fraction Ib; (d) fraction I; (e) affinity eluate.

identical substrate specificities. For fractions I and IV, the activities with 1,2-dichloro-4-nitrobenzene were 8% and 5% respectively of those obtained with 1-chloro-2,4-dinitrobenzene, and with ethacrynic acid the values were 2% and 0.2%. Neither enzyme fraction had detectable activity with *p*-nitrobenzyl chloride, 2,3-epoxy-(*p*-nitrophenoxy)propane, bromosulphophthalein or nitropyridine oxide when these substrates were tested with up to 0.25 mg of enzyme protein. Both types of enzyme could be detected after electrophoresis by using methyl iodide as substrate (Fig. 5), but a quantitative measurement of this activity has not yet been obtained.

Amino acid composition

The amino acid composition of acid hydrolysates of the enzyme IV was determined by standard methods. The results are shown in Table 2.

Amino acid compositions were used to estimate sequence differences and the degrees of relatedness between this enzyme and the transferases A, AA, B and C from rat liver (Jakoby, 1978) by employing the methods of Metzger *et al.* (1968) and Marchalonis & Weltman (1971). On comparison between the insect enzyme and those from rat liver, the parameters $S\Delta Q$ defined by Marchalonis & Weltman (1971) were in the range 60–80, and values for the Difference Index defined by Metzger *et al.* (1968) were in the range 13–14.

Determination of pH optimum

The pH optimum for the enzyme fractions I and

Fig. 4. Subunit composition of glutathione *S*-transferases from *W. cervinata*

Electrophoresis in 12.5% polyacrylamide gel was performed in the presence of 0.1% sodium dodecyl sulphate as described by Laemmli (1970). Samples were as follows: (a) fraction IV; (b) lysozyme oligomers; (c) fraction IV after passage through Sephacryl S-200; (d) fraction IVa; (e) fraction IVb; (f) fraction I.

IV were determined. For both, a broad optimum at pH 7.5–8.5 was observed.

Kinetic studies

The kinetic characteristics of the enzyme IVb were studied. The rate of the enzyme-catalysed reaction varied with substrate concentration in a complex fashion. When the GSH concentration was varied at a fixed concentration of 1-chloro-2,4-dinitrobenzene, the results, plotted by the Eadie–Hofstee method (Eadie, 1942; Hofstee, 1952), indicated markedly non-Michaelian kinetics, the curves being concave-upwards. When the 1-chloro-2,4-dinitrobenzene concentration was varied at a fixed GSH concentration, the $v/[S]$ -versus- v curves were almost vertical and appeared to be slightly curved (see Figs. 6a and 6b).

Both products were found to be inhibitory. The inhibition was analysed graphically by means of Dixon (1953) plots. The conjugate appeared to bind in normal hyperbolic fashion; Dixon plots made with

Table 2. Amino acid composition of glutathione *S*-transferase IV from *Wiseana cervinata*

Amino acid contents were determined as described in the Materials and methods section. The results are expressed as the means of duplicate determinations on two different preparations of the enzyme, with standard deviations. The number of residues per molecule is computed on the basis of an M_r value of 45 600.

Amino acid	Composition (residues/ molecule of protein)
Lysine	36.5 ± 1.0
Histidine	7.0 ± 0.2
Arginine	12.0 ± 1.5
Aspartic acid	32.5 ± 2.5
Threonine	25.9 ± 3.5
Serine	14.8 ± 2
Glutamic acid	35.3 ± 2
Proline	16.2 ± 0.9
Glycine	30.2 ± 0.5
Alanine	26.2 ± 2.0
Cysteic acid	2.7
Valine	28.6 ± 1.1
Methionine	5.0 ± 1.0
Isoleucine	20.82 ± 1.8
Leucine	37.2 ± 0.1
Tyrosine	24.4 ± 0.1
Phenylalanine	25.4 ± 0.4
Tryptophan	Trace

respect to either substrate were linear. On the other hand, Dixon plots made with respect to either substrate when NaCl was the inhibitor were clearly parabolic. If reciprocal velocities were plotted against the square of the chloride concentration, then the plots approximated closely to linearity (Fig. 7). Correlation coefficients ($1/v$ versus $[I]^2$) for these curves were, with one exception, greater than 0.98, and the coefficients of variation on the linear-regression parameters were in the range 0.05–0.1.

Apparent inhibition constants for the conjugate were found to be approx. 30 μ M and 70 μ M when GSH and 1-chloro-2,4-dinitrobenzene respectively were the varied-concentration substrates. The concentration of NaCl giving 50% inhibition was approx. 0.2 M. Eadie–Hofstee curves obtained when NaCl was the inhibitor and 1-chloro-2,4-dinitrobenzene the varied-concentration substrate had a positive gradient (not shown). These curves correspond to the low-velocity part of those shown in Fig. 6 and confirm their non-linear nature.

Discussion

The results presented here confirm and extend the conclusions drawn in a previous study on another insect species, *Galleria melonella* (Chang *et al.*, 1981), namely that the glutathione *S*-transferases from insect sources may resemble quite closely in

Fig. 5. Demonstration of catalytic activity of the major glutathione *S*-transferases from *W. cervinata*

The enzyme samples were subjected to electrophoresis in a polyacrylamide gel slab and the zymogram was developed as described in the Materials and methods section. Enzyme samples were as follows: (a) fraction I (100 μ g); (b) fraction Ia (200 μ g); (c) fraction Ib (300 μ g); (d) fraction IVb (200 μ g).

physical and catalytic properties those that have been isolated from vertebrate sources.

The M_r values of the native enzyme preparations examined in this work (40 000) are consistent with those determined in this department for the glutathione *S*-transferases from a wide variety of sources (Clark & Smith, 1975). They are somewhat lower than those commonly reported in the literature, but this may be a result of the experimental conditions used (Motoyama & Dauterman, 1979). The M_r values determined for the subunits from the IVb and the I preparations (23 000 and 24 500 respectively) are consistent with the values quoted for a variety of vertebrate enzymes (see Jakoby, 1978). It appears that there may be some heterogeneity in the larger-molecular-mass subunit class, since only a single-subunit form is seen, but two physically distinct enzyme proteins (Ia and Ib) are derived from this subunit class. A similar situation is seen with the enzyme forms IVa and IVb, but there is doubt as to whether these forms are physically distinct entities or merely artifacts of the isoelectrofocusing process. All the enzymes from the I and IV groups appear to be homodimers. There has been no evidence for heterodimers being formed *in vivo* between the two classes of subunit.

The immunological experiments indicate that there are two distinct groups of enzymes, probably corresponding to the two classes of subunit. The enzyme activity from peak III was found to be immunologically related to that from peak IV, but

there was insufficient material to ascertain whether or not it possessed the same types of subunit as the enzyme IV species. Previous studies on insects had demonstrated the existence of multiple glutathione *S*-transferases (Clark *et al.*, 1973; Clark & Dauterman, 1982) in these organisms, but the studies were insufficiently detailed to give any idea as to the relationships between these enzymes. The present work shows for the first time that the structural relationships among multiple glutathione *S*-transferases in invertebrate species may be analogous to those holding in, for instance, the white rat. The existence of subunits of differing size and differing immunological properties is compatible with, but does not prove, that these subunits may be derived from differing genetic loci. The possible existence of multiple subunits within a particular size class, as discussed above, is a situation that also has a precedent in the vertebrate situation (Beale *et al.*, 1982; Mannervik & Jansson, 1982).

It may tentatively be concluded that the amino acid composition of the insect enzyme examined shows a relationship to the amino acid compositions of the rat liver enzymes. The values of the $S\Delta Q$ index (Marchalonis & Weltman, 1971) obtained for the comparison of the *Wiseana* IVb enzyme with the rat liver glutathione *S*-transferases A, AA, B and C were in the range 60–80. On the basis of the empirical argument of Marchalonis & Weltman (1971), these values may be taken to indicate similarity, these authors having shown that com-

Fig. 6. Kinetics of glutathione *S*-transferase IVb from *W. cervinata*

Initial reaction velocities at 30°C at pH 6.5 were measured in (a) at various concentrations of 1-chloro-2,4-dinitrobenzene at three fixed concentrations of GSH: ●, 0.5 mM; ○, 1.0 mM; □, 2.0 mM. In (b) velocities were measured over a range of GSH concentrations and at three fixed concentrations of 1-chloro-2,4-dinitrobenzene: ●, 0.2 mM; ○, 0.5 mM; □, 1.0 mM. Velocities are normalized with respect to the volume of enzyme solution used.

parisons between the haemoglobin subunits among different species may generate values as high as 100–120. The values obtained from comparisons amongst the rat liver glutathione *S*-transferases fell into the range 2–56, with a median value of 35. A similar conclusion could be arrived at by using the Difference Index defined by Metzger *et al.* (1968).

When these data are analysed statistically (Cornish-Bowden, 1977), it is not possible to propose statistically significant homology between the insect and the rat liver enzymes, so that the apparent relationship must be regarded with caution.

The kinetics of the enzyme are complex, and it has not yet been possible to obtain a rate equation to fit the data. A number of observations may be made, however. Qualitative examination of the plotting patterns obtained, noting particularly the non-linear *v*-versus-*v*/[GSH] plots (Fig. 6b) and the positive gradients in the *v*-versus-*v*/[1-chloro-2,4-dinitrobenzene] plots (Fig. 6a), conform to patterns predicted by Childs & Bardsley (1976) for 2:2 functions. If this is the case, then the data may be compatible with the steady-state random mechanism proposed for the glutathione *S*-transferase A from rat liver. There are differences in detail between the kinetic patterns obtained with the *Wiseana* enzyme, with the rat liver enzyme and with the transferase isolated from *Galleria mellonella* (Chang *et al.*, 1981). For instance, whereas both insect enzymes exhibit product inhibition by NaCl, the rat liver transferase A does not. Further, the product inhibition seen with the *Galleria* enzyme is characterized by apparently linear plots, in contrast with the parabolic inhibition by NaCl observed in the present study. However, all seem qualitatively to agree with the steady-state random mechanism proposed by Jakobson *et al.* (1979). Insolubility of 1-chloro-2,4-dinitrobenzene limits the concentration range over which the behaviour of the enzyme with regard to this substrate may be studied, and this limits the interpretation of the present work.

We have not yet directly demonstrated a 'ligandin' (Levi *et al.*, 1969; Litwack *et al.*, 1971) character in these proteins. However, the fact that they may be adsorbed with a high degree of specificity on the affinity matrix used and that they may be eluted from it with bromosulphophthalein suggests that they share with the vertebrate enzymes the ability to bind organic anions.

As a whole, the results obtained in the present work suggest that the insect glutathione *S*-transferases are very similar in their properties and in their relationships to one another to those that have been studied in vertebrate organisms. This similarity across a wide evolutionary gap is striking. The glutathione *S*-transferases are enzymes of low specificity for the electrophilic substrate and of low catalytic efficiency (Jakoby, 1978). These properties appear to combine to give a broad detoxification mechanism offering a defence to the organism against a wide variety of toxic chemicals. That the properties of these enzymes are conserved to the extent that now seems apparent indicates the importance of the detoxification role that they have to play.

Fig. 7. Effect of products on the catalytic action of glutathione *S*-transferase IVb from *W. cervinata*. Initial velocities were measured at 30°C at pH 6.5 as described in the Materials and methods section. In (a) and (c) the effect of added *S*-(2,4-dinitrophenyl)-glutathione was examined. In (a) the concentration of GSH was 1 mM and that of 1-chloro-2,4-dinitrobenzene was as follows: ■, 0.05 mM; □, 0.1 mM; ●, 0.2 mM; ○, 1.0 mM. In (c) the concentration of 1-chloro-2,4-dinitrobenzene was 1 mM and that of GSH was as follows: △, 0.05 mM; ■, 0.1 mM; □, 0.2 mM; ●, 0.5 mM; ○, 2.0 mM. In (b) and (d) the effect of added NaCl was examined. In (b) the concentration of GSH was held at 1 mM and that of 1-chloro-2,4-dinitrobenzene was as follows: ■, 0.25 mM; ●, 0.4 mM; □, 0.5 mM; ○, 1.0 mM. In (d) the concentration of 1-chloro-2,4-dinitrobenzene was 1 mM and that of GSH was as follows: ■, 0.4 mM; ●, 0.5 mM; □, 1 mM; ○, 2.0 mM.

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