Cross Specificity in Some Vertebrate and Insect Glutathione-Transferases with Methyl Parathion (Dimethyl *p*-nitrophenyl Phosphorothionate), 1-Chloro-2,4-dinitrobenzene and *S*-Crotonyl-*N*-acetylcysteamine as Substrates

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1. Enzymes catalysing the reaction between GSH and methylparathion (dimethyl *p*-nitrophenyl phosphorothionate), 1-chloro-2,4-dinitrobenzene and *S*-crotonyl-*N*-acetylcysteamine were separated by $(\text{NH}_4)_2\text{SO}_4$ precipitation from homogenates of sheep, rat and mouse livers and from homogenates of cockroaches, houseflies and grass grubs. 2. Electro-focusing of the preparations from each of these species separated a number of zones, each of which catalysed the reaction of GSH with all three substrates. 3. Ion-exchange chromatography on CM-cellulose also separated a number of fractions in which activity towards the three substrates coincided. 4. In both separation methods patterns of the activities were consistent with the presence in all species of several GSH transferases each having a degree of cross specificity towards the three substrates.

A major question in the study of detoxication enzymes is the relation of the enzymes of normal intermediary metabolism to those concerned with the detoxication of xenobiotics. GSH *S*-aryltransferase (Booth *et al.*, 1961; Cohen *et al.*, 1964) is such a detoxication enzyme that acts on unnatural substrates but is nevertheless widely distributed in nature (Smith, 1968) and which is sometimes found in surprisingly high activity (Clark *et al.*, 1967). Other important xenobiotics detoxified by similar GSH-dependent enzymes are lindane (γ -hexachlorocyclohexane) (Bradbury & Standen, 1959; Clark *et al.*, 1969) and some organophosphates (Hutson *et al.*, 1972). The insecticide DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane] is also detoxified in resistant houseflies by a GSH-dependent enzyme that has some similarity to the various GSH transferases and which has been considered to be a possible member of this group (Balabaskaran & Smith, 1970).

The employment of GSH transferases in normal metabolic pathways has received little study though the presence of this type of enzyme is made probable by the occurrence in nature of a number of *S*-substituted glutathione derivatives (Fowden, 1964). These compounds have structures which suggest that they could arise from intermediates of amino acid or fat metabolism. Examples are *S*-(1,2-dicarboxyethyl)-glutathione (Calam & Waley, 1963) and *S*-isopropyl-carboxymethylglutathione (Kuwaki *et al.*, 1963) from animal sources and *S*-(2-methyl-2-carboxyethyl)-glutathione from a plant source (Suzuki *et al.*, 1962).

Speir & Barnsley (1971) have suggested that 2,3-unsaturated acyl-CoA thiol esters might be the nor-

mal intermediary metabolites, which could serve as substrates in the formation of the naturally occurring *S*-substituted glutathiones, and they have partially purified an enzyme which they assayed with model substrates such as *S*-crotonyl-*N*-acetylcysteamine and *S*-crotonylpantetheine. They also pointed out the similarity of this enzyme to the GSH *S*-alkenyltransferase described by Boyland & Chasseaud (1969), which acted on certain xenobiotics containing activated double bonds.

We have assumed the GSH *S*-crotonyl thioester transferase of Speir & Barnsley (1971) to be a representative of GSH transferases with a 'physiological' role in normal metabolism and have compared it with the GSH transferases that act on two non-physiological or xenobiotic substrates, 1-chloro-2,4-dinitrobenzene and methylparathion (dimethyl *p*-nitrophenyl phosphorothionate).

It is shown below that GSH transferases present in vertebrates and insects act preferentially on each of these xenobiotic substrates but that each transferase also acts to some extent on the other xenobiotic as well as on the substrate of GSH *S*-crotonyl thioester transferase.

Materials and Methods

Substrates

S-Crotonyl-*N*-acetylcysteamine was a sample prepared by Speir & Barnsley (1971). 1-Chloro-2,4-dinitrobenzene and diethyl maleate were commercial samples. Methylparathion (dimethyl *p*-nitrophenyl

phosphorothionate, m.p. 36°C) was provided by Monsanto Chemical Co., St. Louis, Mo., U.S.A. Methyl *p*-nitrophenyl thiophosphate was prepared as its diethylmethylamine salt, m.p. 91°C, by the method of Melnikov *et al.* (1961).

Enzyme assays

GSH *S*-crotonyl thioester transferase assays were carried out as described by Speir & Barnsley (1971). Assays were also made with diethyl maleate as described by Boyland & Chasseaud (1967). GSH *S*-aryltransferase was assayed with 1-chloro-2,4-dinitrobenzene as described by Clark *et al.* (1967). Phosphoric acid triester-GSH *S*-methyltransferase (Fukami & Shishido, 1966; Hutson *et al.*, 1972) was assayed with methylparathion as substrate by a method in which the methyl *p*-nitrophenyl thiophosphate produced was measured by a procedure described earlier (Smith & Turbert, 1964) in which reduction of the nitro group was followed by measurement of the alkali-soluble aromatic amino group.

Incubations were at 37°C and were started by adding methyl parathion in 0.05 ml of ethanol to give a 0.3 mM solution in a total volume of 4 ml of enzyme in 0.2 M-Tris-HCl buffer, pH 8, containing 1.5 mM-GSH. After 30 min 2 ml of TiCl₃ (freshly diluted tenfold from a stock solution of 12% w/v, TiCl₃ in 15% w/v, HCl) was added to stop the reaction and the mixture left for 1 h at room temperature. Chloroform (2 ml) and 0.75 M-NaOH (1 ml) were added and the mixture was shaken to extract reduced substrate until the blue colour of Ti(OH)₃ was replaced by a white precipitate. The mixture was centrifuged and 2 ml of the clear supernatant withdrawn to assay the alkali-soluble aromatic amino compounds by mixing with 1 ml of 0.5% *p*-dimethylaminobenzaldehyde in aq. 50% (v/v) acetic acid. The resulting yellow colour was measured after 15 min at 440 nm and compared with a standard curve prepared from standard solutions of methyl *p*-nitrophenyl thiophosphate.

Control incubations were carried out omitting GSH and also with the enzyme solution replaced with buffer. Recovery of added methyl *p*-nitrophenyl thiophosphate from 0.01–0.1 mM solutions by this method was within ±3% both from pure solutions and from solutions containing protein equivalent to 100 mg in the standard assay incubation mixture.

Enzyme preparations

Preliminary experiments showed that the enzyme activities were lost during storage of crude homogenates of either insects or liver but that (NH₄)₂SO₄-precipitated enzymes could be stored at –15°C without loss of activity and could be redissolved to give solutions which were stable for the time required for electrofocusing.

Freshly removed livers from rats, mice or sheep were chilled to 0°C and homogenized with 4 vol. of water in an MSE Ato-Mix blender. The homogenates were centrifuged at 10000g at 5°C for 30 min and the supernatants treated at 5°C with finely powdered (NH₄)₂SO₄. The protein that was precipitated between 50 and 100% saturation was centrifuged off and stored at –15°C. Before use the precipitate was dissolved in water and dialysed overnight against tap water.

Houseflies (*Musca domestica*) and cockroaches (*Periplaneta americana*) were similarly homogenized in 4 vol. of water and the 10000g supernatant was treated with (NH₄)₂SO₄ at 5°C. Precipitates were collected between 50 and 70% saturation, stored at –15°C and dissolved in water and dialysed as above before use.

Grass grubs (*Costelytra zealandica*) were treated similarly except that a 50–60%-satd.-(NH₄)₂SO₄ fraction was collected.

Some fractionation of the three enzyme activities and considerable loss of activity resulted from this partial purification and only about a third of the activity towards 1-chloro-2,4-dinitrobenzene and *S*-crotonyl-*N*-acetylcysteamine in the 10000g supernatants remained in the fractions used for subsequent work. Activity towards methylparathion in the (NH₄)₂SO₄ precipitate was usually about one-fifth of the activity of the crude homogenate and further losses occurred during dialysis.

Isoelectric focusing

This was carried out at 5°C in Ampholine buffers (LKB Producter AB, Stockholm, Sweden) as described by Goodchild & Smith (1970) and eluted fractions (3 ml) were assayed for enzyme activity. Focusing periods were between 36 h and 3 days but no significant difference in the patterns was seen with the different times. At least three runs were made with each source of enzyme and isoelectric points of fractions with peak activity were reproducible to ±0.1 pH units on different occasions although some variation was found in the relative amounts of enzyme activity associated with the various peaks on different occasions.

Ion-exchange chromatography

CM-23 cellulose (Reeve Angel and Co. Ltd., London E.C.4, U.K.) was pretreated as described by Petersen & Sober (1962) and equilibrated with sodium phosphate buffer, pH 6.25 (*I* 0.01), before packing in a column (40 cm × 1.5 cm). The dialysed enzyme (5–6 ml) was applied to the column and washed through with 30 ml of phosphate buffer, pH 6.5 (*I* 0.01), and then with a gradient of NaCl in the same buffer (*I* 0.01–1.0)

or with increasing buffer concentration (10.01–0.2) over 300ml. In some experiments 150ml of buffer (10.01) was passed through the column before starting the gradient. No activity was eluted in this period and subsequent elution of activity occurred at the same value of I as under standard conditions.

Enzyme assays were carried out on fractions (3 ml) eluted from the columns and protein contents of the fractions assessed by measurement of E_{280} with bovine serum albumin as standard. All ion-exchange experiments were carried out at least three times.

Results

Sheep liver enzyme

The dialysed enzyme was focused in ampholine buffer, pH 7–9, as described above and the eluates

were analysed for activity towards the three substrates. Two major activity-zones were found, one containing most of the 1-chloro-2,4-dinitrobenzene and *S*-crotonyl-*N*-acetylcysteamine activity and the other containing the bulk of the activity towards methylparathion (Fig. 1). In one experiment the fractions were also assayed with diethyl maleate by the procedure of Boyland & Chasseaud (1967) for GSH *S*-diethyl maleate transferase and a plot of this activity in each fraction coincided with the plot of the activity towards *S*-crotonyl-*N*-acetylcysteamine though the activity towards diethyl maleate was low and in the peak fraction the ratio of activities was 32:1 in favour of the former substrate.

Fractions 10–15 from the column shown in Fig. 1(a) were pooled and refocused in Ampholine buffers, pH 6–8. A major peak of activity with respect to both

Fig. 1. Isoelectric focusing of GSH transferases

An $(\text{NH}_4)_2\text{SO}_4$ -precipitated fraction of liver or insect homogenates prepared as described in the text was focused in ampholine buffers. After 2 days at 5°C, fractions (3 ml) were eluted and assayed for enzyme activity with *S*-crotonyl-*N*-acetylcysteamine (●), 1-chloro-2,4-dinitrobenzene (□) and methylparathion (Δ). —, Plots of pH against fraction number. Enzyme sources were (a) sheep liver; (b) rat liver; (c) grass grubs; (d) fractions 14–24 from (c) refocused. The enzyme activity values given are correct for 2,4-dinitrobenzene but for *S*-crotonyl-*N*-acetylcysteamine the values are (a) $\times 6$, (b) $\times 5$, (c) 4×10^2 and (d) 2.5×10^2 the activities found; for methylparathion the values are (a) 5×10^2 , (b) 5×10^3 , (c) 4×10^4 and (d) 1.2×10^4 the activities found. Enzyme activity units are $\mu\text{mol/min}$.

Table 1. *Michaelis constants of electrofocused sheep liver enzyme activities*

Enzymes used were fractions 10–15 and 30–34 from an experiment similar to that shown in Fig. 1a. K_m values of GSH were determined from double-reciprocal plots obtained at GSH concentrations between 0.2–2.0 mM with 0.15 mM-*S*-crotonyl-*N*-acetylcysteamine or 0.1 mM-1-chloro-2,4-dinitrobenzene. In determination of K_m values for the second substrate, concentrations of *S*-crotonyl-*N*-acetylcysteamine were between 0.05–0.25 mM in 0.5 mM-GSH and concentrations of 1-chloro-2,4-dinitrobenzene were between 0.02–0.2 mM in 1 mM-GSH.

Enzymes from	Michaelis constants (K_m) for substrates			
	GSH with 0.1 mM-1-chloro-2,4-dinitrobenzene	GSH with 0.15 mM- <i>S</i> -crotonyl- <i>N</i> -acetylcysteamine	1-chloro-2,4-dinitrobenzene in 1 mM-GSH	<i>S</i> -crotonyl- <i>N</i> -acetylcysteamine in 0.5 mM-GSH
pI 7.15 zone	0.24, 0.33	0.22	0.76, 0.97	0.37
pI 9.4–9.5 zone	0.71, 0.59	—	0.08, 0.08	—

1-chloro-2,4-dinitrobenzene and *S*-crotonyl-*N*-acetylcysteamine was found at pH 7.15. Only a small peak of activity for the methyl parathion enzyme was observed associated with the pH 7.15-isoelectric fraction but a small peak of activity towards this substrate was isoelectric at pH 6 where a small peak for 1-chloro-2,4-dinitrobenzene was also present.

Fractions 30–34 from the column shown in Fig. 1(a) were similarly refocused in pH 8–10 ampholine buffer. Peak concentrations of the methyl parathion activity were found at pH 9.2, 9.4 and 9.5 which were each associated with small peak activities towards 1-chloro-2,4-dinitrobenzene. No enzymic activity was measurable when *S*-crotonyl-*N*-acetylcysteamine was used as substrate.

In an experiment similar to that of Fig. 1(a) the fractions from each of the two major zones were pooled and used to determine the Michaelis constants for GSH *S*-aryl transferase and GSH *S*-crotonyl thioester transferase. Table 1 shows that the K_m values for GSH with 1-chloro-2,4-dinitrobenzene (0.1 mM) and *S*-crotonyl-*N*-acetylcysteamine (0.15 mM) were similar for the activities isoelectric at pH 7.15 but the K_m constants for both GSH and 1-chloro-2,4-dinitrobenzene in the pI 7.15 and pI 9.4 zones differed considerably in assays of GSH *S*-aryltransferase. Moreover, in determining the K_m values, experiments with the GSH *S*-aryltransferase activity from the more alkaline isoelectric zone gave double-reciprocal plots that were non-linear at the higher GSH concentrations and suggested that substrate inhibition of this enzyme occurred in concentrations greater than 1 mM-GSH.

A sample of the dialysed sheep liver enzyme was equilibrated with 0.01 M-sodium phosphate buffer and fractionated by ion-exchange chromatography at pH 6.5 on CM-cellulose as described above. Fractions were assayed with the three substrates and three

activity peaks were found (Fig. 2). The first of these was not retained by the column and contained enzymes acting on all three substrates. The second which was eluted from CM-cellulose at *I* 0.06 also had all three activities but the peak eluted at *I* 0.08 contained methyl parathion activity with only a little 1-chloro-2,4-dinitrobenzene activity.

In a similar experiment the portion containing all three activities, was not retained on the CM-cellulose column at pH 6.5 was dialysed and then electrofocused in ampholine buffers, pH 6–10. Only the enzymes with pI 7.15 were detected in this experiment and the ratio of *S*-crotonyl-*N*-acetylcysteamine activity (5 μ mol/min per fraction) to 1-chloro-2,4-dinitrobenzene activity (40 μ mol/min per fraction) in the peak fraction was similar to that present in enzymes focused at pH 7.15 without preliminary ion-exchange fractionation (Table 2).

The fractions containing activity towards methyl parathion eluted from the CM-cellulose column between *I* 0.05 and *I* 0.10 were also dialysed and electrofocused in Ampholine buffers, pH 6–10. No methylparathion activity was measurable after dialysis but the major zone which focused at pH 6.9 had both 1-chloro-2,4-dinitrobenzene activity (6 μ mol/min in the peak activity fraction) and some *S*-crotonyl-*N*-acetylcysteamine activity (0.7 μ mol/min in the peak activity fraction). A small activity peak containing only 1-chloro-2,4-dinitrobenzene activity was present with pI 9.4 (1 μ mol/min in the peak fraction).

In a converse experiment the electrofocused enzymes contained in the fractions corresponding to 10–15 of Fig. 1(a) were pooled, dialysed and separated on CM-cellulose at pH 6.5 as described above. No methyl transferase activity was measurable after dialysis but three peak activities with aryltransferase and alkenetransferase were found, one of which was not retained by the column. A second peak with

Fig. 2. Ion-exchange separation of GSH transferases from sheep liver

An $(\text{NH}_4)_2\text{SO}_4$ -precipitated fraction of liver was dissolved in phosphate buffer, pH6.5 (I 0.01), and chromatographed on a column of CM-cellulose as described in the text. A gradient of NaCl in the same buffer was started at fraction 22 and fractions (3 ml) of the eluate were analysed for activity towards *S*-crotonyl-*N*-acetylcysteamine (●), 1-chloro-2,4-dinitrobenzene (□) and methylparathion (Δ). —, Plot of the NaCl gradient. The enzyme activity values shown are correct for aryltransferase but for methyltransferase the values should be $\times 10^2$ and for crotonyl transferase $\times 10$ the activities given. Enzyme activity units are $\mu\text{ mol/min}$.

Table 2. Ratios of specific activities towards different substrates of separated GSH transferases from sheep liver

Enzymes were (a) isoelectric-focused fractions near pI7.1 similar to fractions 10–15 of Fig. 1(a). (b) Focused fractions near pI9.5 similar to fractions 30–34 of Fig. 1a. (c) Material not retained on a CM-cellulose column at pH6.5 and I 0.01. Substrates were *S*-crotonyl-*N*-acetylcysteamine (A), 1-chloro-2,4-dinitrobenzene (B) and methylparathion (C) and assays were made as described in the text. Values are means of the number of experiments made with range shown in parentheses.

Enzyme source	Ratio of specific activities	
	B/A	B/C
(a) Electrofocused, pI7.1	7.1 (5.2–10.0) (5 expts.)	3800 (3000–5000) (5 expts.)
(b) Electrofocused, pI 9.5	* very large (3 expts.)	200 (180–230) (3 expts.)
(c) Not retained on CM-cellulose	10.0 (7.7–11.1) (4 expts.)	4000 (2 expts.)

* Activity towards *S*-crotonyl-*N*-acetylcysteamine not measurable.

about the same number of units was eluted at I 0.06 and a third minor peak also containing both *S*-crotonyl-*N*-acetylcysteamine and 1-chloro-2,4-dinitrobenzene activity was eluted at I 0.03.

Rat liver enzyme

The 60–100%-satd.-($\text{NH}_4)_2\text{SO}_4$ precipitate from rat liver was electrofocused in ampholine buffers,

pH6–10. Three major zones with similar ratios of activities towards 1-chloro-2,4-dinitrobenzene and *S*-crotonyl-*N*-acetylcysteamine were present with a further zone containing most of the activity towards methyl parathion and some 1-chloro-2,4-dinitrobenzene activity (Fig. 1b and Table 3). The enzyme was also fractionated on CM-cellulose at pH6.5 by elution with a salt gradient of I 0.01–1.0 over 300ml. Table 4 shows that a small zone containing the three

enzyme activities was not retained by the column and two peaks containing mainly methylparathion activity were eluted at *I* 0.2–0.23. A further major peak of this activity that eluted at *I* 0.25 was associated with larger quantities of 1-chloro-2,4-dinitrobenzene and *S*-crotonyl-*N*-acetylcysteamine activity (Table 4).

Fractions containing the three peaks that were eluted from the column between *I* 0.1 and *I* 0.3 were pooled, dialysed and electrofocused between pH 6–10. No activity towards methyl parathion remained after dialysis but the isoelectric points of the peak activity towards 1-chloro-2,4-dinitrobenzene and *S*-crotonyl-*N*-acetylcysteamine were identical with those obtained on direct focusing of the enzyme. The pattern of the plot of these values was similar to Fig. 1(b) except that the inflexion in the curve at pI 8.1 now appeared as a fully resolved peak at this pH.

Mouse liver enzyme

Electrofocusing in Ampholine buffers (pH 6–10) of the (NH₄)₂SO₄-precipitated enzyme showed two major peaks with the three assayed activities present in both peaks (Table 3) though the focused fraction at pH 8.2 contained much more 1-chloro-2,4-dinitro-

benzene and *S*-crotonyl-*N*-acetylcysteamine activity; the predominant activity present in the pH 9.8-focused fraction was towards methyl parathion. Chromatography on CM-cellulose was very similar to that in the rat, a small peak containing less than 5% of the total 1-chloro-2,4-dinitrobenzene and *S*-crotonyl-*N*-acetylcysteamine activity not being retained by the column and enzymes acting on all three substrates eluting at *I* 0.2 preceded by a small peak containing relatively more of the methylparathion activity at *I* 0.18 (Table 4).

Housefly enzyme

The 50–70%-satd.-(NH₄)₂SO₄ precipitate was electrofocused in Ampholine buffers, pH 4–8. Coincidence of peaks for 1-chloro-2,4-dinitrobenzene and *S*-crotonyl-*N*-acetylcysteamine was observed though only the peak of methylparathion activity with pI 6.8 coincided exactly with the activity towards the other two substrates (Table 3). Though the peak of activity for these substrates at pI 5.1 contained much methylparathion activity, the main peak of activity towards this substrate was found at pI 4.8. Most of the three housefly enzyme activities were not

Table 3. *Distribution of GSH transferases between electrofocused fractions of enzymes from insects and livers*

Partially purified enzyme mixtures were focused in Ampholine buffer with the pH ranges shown. After 36 h the contents of the apparatus were run out and zone fractions (3 ml) collected. Assays were made with *S*-crotonyl-*N*-acetyl cysteamine (A), 1-chloro-2,4-dinitrobenzene (B) and methylparathion (C) as described in the text. Peak pH values quoted are peaks of activity plotted as in Fig. 1, and activities in each zone are expressed as % of the activity loaded into the focusing apparatus.

Source of enzyme and buffer range	Fractions eluted	Peak pH	Enzyme activity (% of loaded enzyme activity recovered with substrate)		
			A	B	C
Sheep liver, pH 7–9	10–15	7.1	68	38	20
	30–34	9.5	0.5	41	72
Rat liver, pH 6–10	14–15	7.6	12	12	3
	18–19	8.0	20	22	4
	24–25	8.6	18	21	3
	30–32	9.4	1	15	27
	20–22	8.2	74	62	3
Mouse liver, pH 6–10	32–34	9.8	1	2	55
Housefly, pH 4–8	10–13	5.1	53	35	32*
	16–18	5.9	17	18	5
	23–25	6.8	16	19	15
	9–12	4.5	24	9	20
Cockroach, pH 3–10	20–22	6.6	64	73	47
	28–30	8.5	8	6	26
	14–17	4.5	12	21	19
Grass grub, pH 3–10	19–22	5.2	28	6	27
	30–33	8.8	2	22	1

* Isoelectric point 4.8 not resolved from the 5.1 peak, assay includes both peaks.

Table 4. *Ion-exchange fractionation of some liver GSH transferases*

Partially purified liver homogenates were chromatographed on CM-cellulose at pH 6.5 in phosphate buffer (I 0.01) as described in the text. A gradient of NaCl in the same buffer was used to elute material retained on the column in the buffer with I 0.01. Enzymes were assayed with the substrates *S*-crotonyl-*N*-acetylcysteamine (A), 1-chloro-2,4-dinitrobenzene (B) and methylparathion (C) as in Table 1 and Fig. 1 and as described in the text. Values quoted are % of enzyme units loaded on the column which were recovered in eluates of I values shown. In similar experiments with insects no significant activity was retained by the column at I 0.01.

Source of enzyme	Eluted fraction no.	I values of peak fractions	Enzyme activity (% of loaded activity recovered with substrate)		
			A	B	C
Sheep liver	13-16	0.01	48	41	10
	46-48	0.06	20	12	13
	52-58	0.10	2	2	6
Rat liver	15-20	0.01	4	5	2
	40-45	0.23	10	10	24
	45-50	0.25	42	58	18
Mouse liver	16-20	0.01	3	2	0
	40-45	0.18	6	8	7
	45-49	0.20	50	64	20

retained on CM-cellulose under the conditions used for the vertebrate enzymes; small amounts of activity were eluted in the NaCl gradient but these were too small to be assayed reliably.

A DDT-resistant strain of housefly was also examined but the electrofocusing pattern and ion-exchange behaviour was similar, differences being within the variation experienced with patterns obtained from susceptible houseflies on different occasions.

Cockroach enzyme

The 40-70%-satd.-(NH₄)₂SO₄ precipitate from a cockroach homogenate was electrofocused in Ampholine buffers, pH 3-10. Three zones of enzyme activity were present (Table 3) in which there was coincidence of the major peaks of activity for the three substrates studied. Ion-exchange chromatography on CM-cellulose at pH 6.5 separated a major peak of activity responding with all three substrates, which was not retained on the column from a minor peak eluted at I 0.1 which contained less than 5% of the activities loaded on the column.

Grass grub enzyme

The 50-63%-satd.-(NH₄)₂SO₄ precipitate from a homogenate of grass grubs was electrofocused in Ampholine buffer, pH 3-10. Three major zones were found and coincidence of the three enzyme activities found in two of these (Fig. 1c). The third with pI 8.3 contained very large amounts of aryltransferase activity and small amounts of the other two enzymes

(800, 0.2 and 0.02 μ mol/min in the pI 8.3 peak fraction with 1-chloro-2,4-dinitrobenzene, *S*-crotonyl-*N*-acetylcysteamine and methylparathion respectively). Fractions 14-24 from the experiment shown in Fig. 1(c) were eluted and refocused between pH 4-6 (Fig. 1d); two peaks at pH 5.1 and 5.5 were found with peak activity towards each of the three substrates used but the major aryl transferase activity peak had little cross specificity to the other two substrates.

The (NH₄)₂SO₄-precipitated enzyme was not retained on CM-cellulose at pH 6.5 under the conditions described above but similar fractionation on DEAE-cellulose at pH 6.3 with elution in a NaCl gradient separated a major peak containing *S*-crotonyl-*N*-acetylcysteamine and 1-chloro-2,4-dinitrobenzene activity which was not retained on the column from two minor zones with activity towards each of these substrates singly which were eluted by the NaCl gradient. No methylparathion activity was detected in the eluates of the DEAE columns.

Discussion

Each of the enzyme activities measured was present in the enzyme extract used for electrofocusing in a number of forms having different isoelectric points. A limited number of these isoenzymes in each species accounted for the bulk of the activity of the particular enzyme measured but, especially in the narrower pH-range experiments (e.g. Fig. 1d), many small peaks were detected. It is not known whether these represent other GSH transferases having only slight cross specificity with the substrates or whether they are

artifacts produced by partial degradation of proteins from the major zones. Such minor artifacts have been reported in electrofocusing experiments with other proteins and enzymes (Carlstrom & Vesterberg, 1967; Flatmark, 1966).

The major peaks of activity were reproducible in experiments with the same source of enzyme carried out on different occasions and the pattern of these main peaks was not affected by the duration of the focusing experiment as had been found earlier for DDT dehydrochlorinase (Goodchild & Smith, 1970). Some evidence for the presence of multiple forms of the enzyme has also been reported for the aryl transferase activity in houseflies by Ishida (1968).

In each of the species examined, coincidence of activity towards the three substrates used was observed in the major electrofocused peaks though relative concentrations in different peaks varied widely. In the vertebrate liver enzymes a minor proportion of the GSH aryl transferase, as assayed by 1-chloro-2,4-dinitrobenzene, was associated with the methyl transferase assayed with methyl parathion but the major proportion of the aryltransferase activity was associated with GSH *S*-crotonyl thioester transferase (Table 3). In one experiment diethyl maleate was used as substrate and though this substrate reacted much more slowly with GSH than *S*-crotonyl-*N*-acetylcysteamine, the coincidence of activity to both these substrates and 1-chloro-2,4-dinitrobenzene at pI 7.1 in sheep suggests that these three compounds are all substrates for the same enzyme. This belief is strengthened by the observation that the K_m values for GSH were similar when either *S*-crotonyl-*N*-acetylcysteamine or 1-chloro-2,4-dinitrobenzene was used as substrate for the enzyme focused at pI 7.1.

A similar situation is found in mouse liver and particularly in rat livers where three isoenzymes of the GSH *S*-crotonyl thioester transferase are exactly paralleled in the plots (Fig. 1*b*) by 1-chloro-2,4-dinitrobenzene activity, and the rest of the 'aryltransferase' activity is associated with the methylparathion activity.

The situation in the flies and cockroaches is similar in that both the methyl transferase and crotonyl thioester transferase activities are associated with some aryl transferase activity. In grass grubs however, the very large amounts of aryltransferase activity obscure the situation unless focusing is carried out over narrow ranges (Fig. 1*d*). Although some isoelectric zones with modest activities to each of the three substrates coincide, there are also two major 1-chloro-2,4-dinitrobenzene activities where the cross specificity towards the other substrates is less obvious.

In most of the species examined the enzymes may be regarded as either crotonyl thioester transferases or methyltransferases having some incidental activity towards 1-chloro-2,4-dinitrobenzene and it is tempting to regard this compound as a general substrate

which may function more or less effectively with any GSH transferase. With this substrate very high specific activities are usually encountered and the non-enzymic blank reaction is also rapid. If the peaks of aryl transferase activity are in fact due to the general response of 1-chloro-2,4-dinitrobenzene to any GSH transferase, as the cross specificity toward the methyl- and crotonyl thioester transferases seems to suggest, the presence of the two very-high-activity aryltransferase peaks in grass grubs is of particular interest since it might indicate a high content of some as yet unidentified GSH transferase.

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