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Multiple Forms of Rat Liver Glutathione *S*-Transferases: Specificity for Conjugation of *O*-Alkyl and *O*-Aryl Groups of Organophosphorus Insecticides

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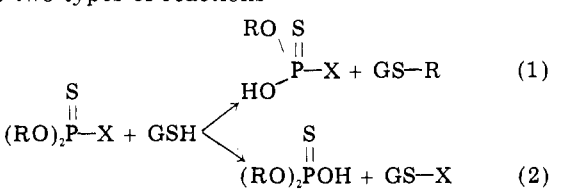
Multiple forms of glutathione *S*-transferases were partially purified from rat liver, using 1-chloro-2,4-dinitrobenzene as the substrate to monitor activity. Their properties were studied, especially with regard to the specificity for *O*-alkyl and *O*-aryl conjugations of organophosphorus insecticides. Substrates studied were diazinon and methyl parathion as well as 3,4-dichloronitrobenzene and methyl iodide. Based upon the nature of the binding to a DEAE–cellulose column, pH–activity relationships, and preference for alkyl and aryl conjugations, the rat liver enzymes were classified into two major groups, each of which was further separated on CM–cellulose and hydroxylapatite columns. The multiple forms showed distinctive or overlapping properties. Their identity with those reported previously is discussed.

Glutathione *S*-transferases (EC 2.5.1.18) are involved in the metabolism of xenobiotics as well as in the binding of diversified groups of chemicals (see reviews by Arias et al., 1976; Jakoby et al., 1976a,b; Jakoby and Keen, 1977). These enzymes are also responsible for the detoxification of certain pesticides (Yang, 1976). With organophosphorus (OP) insecticides as substrates, glutathione *S*-transferases catalyze two types of reactions



where R = alkyl, X = "leaving group" and aryl group.

Whether the alkyl conjugation (eq 1) and the "leaving group" conjugation (eq 2) are catalyzed by the same enzyme has been a question for many years (Yang, 1976). In the case of rat liver supernatant, Hollingworth et al. (1973) reported that two distinct enzymes were involved in the two reactions. In contrast, Motoyama and Dauterman (1977) reported that both types of reactions were catalyzed by the same housefly glutathione *S*-transferase and they found that the reaction ratio varied markedly depending upon the alkyl and the "leaving group" structures of the OP substrates. However, Usui et al. (1977a) purified several glutathione *S*-transferases from the fat body of the the American cockroach and demonstrated that these enzymes had overlapping specificities. The same workers (Usui et al., 1977b) also separated several forms of glutathione *S*-transferases from rat liver, which showed overlapping specificities for the OP insecticides. Although not yet established, it appears that the mode of interaction between glutathione *S*-transferases and OP insecticides varies according to the structure of the substrates as well as the source and form of the enzymes.

The present study was undertaken in an attempt to clarify the apparent contradictions. Multiple forms of rat liver glutathione *S*-transferases were separated and their properties were studied especially with emphasis on alkyl and aryl group conjugations.

METHODS AND MATERIALS

Chemicals. DEAE–cellulose (DE52) and CM–cellulose were obtained from Pharmacia Fine Chemicals. Hydroxylapatite (Bio-Gel HTP) was obtained from Bio-Rad Laboratories. Precoated silica gel plates (Polygram Sil N-HR) (0.25 mm) for the thin-layer chromatography were obtained from Brinkmann Instruments. Sources of the substrates used were as follows: 1-chloro-2,4-dinitrobenzene (DNCB) from Aldrich Chemical Co., 1,2-dichloro-4-nitrobenzene (DCNB) from Eastman-Kodak,

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[^{14}C]methyl iodide (25 mCi/mmol) from Amersham/Searle Corp., reduced glutathione (GSH) from ICN Pharmaceuticals. Methyl [^{14}C]parathion [*O,O*-dimethyl *O*-*p*-nitrophenyl-2,6-^{14}*C* phosphorothioate] (6.0 mCi/mmol) was synthesized as described previously (Motoyama and Dauterman, 1977). [^{14}C]Diazinon [*O,O*-diethyl *O*-(2-isopropyl-4-methylpyrimidin-2-^{14}*C*-6-yl) phosphorothioate] (1.1 mCi/mol) was a gift from Ciba-Geigy Corp.

Enzyme Assay. Glutathione *S*-transferases activity for DCNB was measured according to the method described by Booth et al. (1961). Fifty microliters of enzyme solution was incubated with 4 mM GSH, 0.4 mM DCNB, and 0.05 M Tris–HCl, pH 9.0, in a total volume of 3.0 mL at 37 °C for 5 min. The change in absorbance was determined at 344 nm and was converted to nanomoles conjugated using the extinction coefficient reported by Askelöf et al. (1975). The activity for methyl iodide conjugation was assayed according to the method of Johnson (1966). The enzyme solution (200 μL) was incubated with 4 mM GSH, 2.4 mM [^{14}C]methyl iodide, and 0.05 M Tris–HCl, pH 8.0, in a total volume of 1.0 mL at 25 °C for 10 min. Activity for DNCB was determined as described by Habig et al. (1974b).

For insecticidal substrates, a reaction mixture contained 4 mM GSH, 0.24 mM [^{14}C]diazinon, or 0.25 mM methyl [^{14}C]parathion, 200 μL of the purified enzyme solution, and 0.05 M Tris–HCl, pH 8.0 buffer in a total volume of 1.0 mL. The mixture was incubated at 37 °C for 1 h and 1 mL of chloroform was added to stop the reaction and to extract the unreacted organophosphorus substrates. The reaction tubes were centrifuged at 500 rpm for 10 min to separate the aqueous and chloroform layers. A reaction mixture without the enzyme was treated similarly and used as a control. The radioactivity in the aqueous layer was quantitated with a Packard Tri-Carb Scintillation Spectrometer using a Triton X-100 cocktail (Patterson and Greene, 1965).

Thin-Layer Chromatography. Thin-layer chromatography (TLC) was used to separate and quantitate the reaction products of organophosphorus substrates. A 100-μL aliquot of the aqueous layer was applied to a 5 × 20 cm silica gel N-HR (precoated 0.25 mm) plate and developed in acetonitrile–water (85:15, v/v) to a height of 10 cm. After drying the plates at room temperature, the same plates were developed in the same direction in hexane–ethyl acetate–benzene (4:2:1, v/v) to a height of 15 cm. Standards were visualized at 254 nm, by iodine vapors, or by 2,6-dibromoquinone-4-chloro-*p*-quinonimine. The radioactivity on the TLC plates were detected using a Packard Radiochromatogram Scanner, and radioactive areas were scraped off the plate and quantified with a Scintillation Counter as described above.

Protein Determination. Total protein was determined according to the method of Sedmak and Gossberg (1977) using bovine plasma albumin for calibration.

Effect of pH on DNCB Activity. An enzyme solution (2–25 μL) was incubated with 1 mM GSH, 1 mM DNCB, and 0.1 M sodium phosphate buffer, pH ranging from 6.2 to 8.0 at 0.2 pH intervals, in a total volume of 3.0 mL. The change in absorbance at 340 nm was recorded at 25 °C for 2.5 min and the equivalent in micromoles was calculated using the extinction coeffecient (E = 916 mM^{-1} cm^{-1}) (Habig et al., 1974b).

Preparation of Enzymes. A slight modification of the procedure described by Habig et al. (1974b) were used in order to obtain multiple forms of rat liver glutathione *S*-transferases. Throughout the purification, the enzyme activity was monitored using DNCB as the substrate. It has been reported that all forms of glutathione *S*-transferases from rat liver are active toward this substrate (Jakoby et al., 1976a; Jakoby and Keen, 1977). For CM cellulose chromatography, [^{14}C]methyl iodide was also used to monitor the active fractions.

Step 1. Livers (12 g) from 100–150-g male, Duplin white rats were homogenized in 38 mL of distilled water using a Sorvall Omni Mixer. The homogenate was centrifuged at 10 000*g* for 1.5 h, and the supernatant was filtered through glass wool.

Step 2. The supernatant was applied to a DEAE–cellulose column (2.5 × 20 cm equilibrated with 10 mM Tris–HCl, pH 8.0) and washed with 250 mL of the same buffer. This eluted the first enzyme peak which was designated fraction I. Then a linear gradient between 150 mL of the initial buffer and 150 mL of the buffer containing 0.5 M KCl was applied. This eluted the second enzyme peak which was designated fraction II. Approximately 4-mL aliquots were collected.

Step 3. Ammonium sulfate was added to 90% saturation, and the proteins precipitated were collected by centrifugation at 10 000*g* for 30 min. Precipitates of enzyme fractions I and II were dissolved in 5 and 7 mL, respectively, of 10 mM potassium phosphate, pH 6.7 (buffer A), and dialyzed for 1 day against three changes of 500 mL of buffer A.

Step 4. Each dialysate was applied separately on a CM–cellulose column (2.5 × 20 cm, equilibrated with buffer A). After washing the column with 250 mL of buffer A, a linear gradient of 150 mL of buffer A and 150 mL of buffer A containing 75 mM KCl was developed. Approximately 4-mL fractions were collected. Four active peaks from fraction I (DEAE–cellulose unbound) and five active peaks from fraction II (DEAE–cellulose bound) were separated. They were designated alphabetically according to the order of elution. Each peak was concentrated by ultra-filtration through Diaflo PM-10 membrane and dialyzed overnight against 500 mL of 10 mM potassium phosphate, pH 6.7, containing 30% glycerol, 2 mM GSH, and 0.1 mM EDTA (buffer B).

Step 5. Each dialysate, except Ib and IIb of which the yield was so small, was applied separately on a hydroxylapatite column (2.5 × 10 cm equilibrated with buffer B). The column was washed with 100 mL of buffer B, and then a linear gradient between 150 mL of buffer B and 150 mL of 400 mM potassium phosphate, pH 6.7, containing 30% glycerol, 2 mM GSH, and 0.1 mM EDTA was applied. Four-milliliter fractions were collected. Active peaks were concentrated by ultrafiltration and stored frozen at −15 °C.

RESULTS AND DISCUSSION

Separation of Multiple Forms. DEAE–cellulose chromatography (step 2) eluted the glutathione *S*-transferases from the rat liver supernatant into two widely separated peaks. The first peak, fraction I which eluted through the column without being bound, accounted for 40% of the initial activity. Figures 1 and 2 illustrates the further resolution of the fraction I and fraction II enzymes into four and five forms, respectively, on CM–cellulose chromatography (step 4). Although the yields of Ib and IIb were extremely small when assayed with DNCB, they were collected as separate peaks because of their activity for methyl iodide. No further separation was obtained on hydroxylapatite chromatography (step 5), except that Ia appeared to be separated into two small peaks. Therefore, the peaks were collected separately and designated as Ia_1 and Ia_2. Since the amount of Ib and IIb obtained after CM–cellulose chromatography was so low, the peaks were not applied on a hydroxylapatite column. The procedure

Table I. Purification of Multiple Forms of Rat Liver Glutathione S-Transferases[a]

procedure	vol, mL	total units	units/ mg of protein	yield, %	purification (fold)
1. 10 000g Sup	37	569.8	0.860	100	1
2. DEAE-cellulose					
I	47	228.9	4.985	40.2	5.8
II	58	268.5	3.355	47.1	3.9
3. AS-90% precipitate					
I	7.7	194.0	4.865	34.0	5.7
II	11.6	228.0	3.756	40.0	4.4
4. CM-cellulose					
Ia	3.0	11.4	6.333	2.0	7.4
Ib	1.8	3.1	8.614	0.5	10.0
Ic	2.8	71.7	9.176	12.3	10.7
Id	3.3	57.1	10.813	10.0	12.6
IIa	3.8	49.8	1.955	8.7	2.3
IIb	3.4	10.5	6.652	1.8	7.7
IIc	3.5	83.3	14.512	14.6	16.9
IId	4.3	37.8	11.286	6.6	13.1
IIe	3.0	17.8	8.985	3.1	10.4
5. hydroxylapatite					
Ia_1	7.0	3.2	5.590	0.6	6.5
Ia_2	6.7	3.5	5.212	0.6	6.1
Ic	6.8	53.5	12.939	9.4	15.0
Id	8.2	47.6	13.712	8.4	15.9
IIa	7.2	40.8	1.954	7.2	2.3
IIc	6.8	77.5	19.128	13.6	22.2
IId	8.2	34.3	15.481	6.0	18.0
IIe	4.5	14.3	15.071	2.5	17.5

[a] Protein concentration was determined by Coomassie Brilliant Blue method (Sedmak and Gossberg, 1977). Unit of activity is defined as 1 μ mol of DNCB conjugated with GSH per min at 25 °C.

for the preparation of the enzyme fractions are summarized in Table I. The purification varied from 2.3-fold for the IIa to 22.2-fold for the IIc. Judging from the total activity for DNCB as the substrate, Ic, Id, IIa, IIc, and IIb were the major forms and Ia_1, Ia_2, Ib, IIb, and IIe were the minor forms. In order to study the specificity toward other substrates, the minor forms were also characterized. The combined yield of all these forms was approximately 50%.

Activity toward Noninsecticidal Substrates. Since methyl iodide and DCNB are widely used substrates for glutathione S-transferases, the activities of the multiple forms of the rat liver enzymes were examined with these compounds. Results with methyl iodide are presented in Table II as nanomol of GS-methyl (mg of protein^{-1}) min^{-1} (specific activity) and total nanomole of GS-methyl/minute (total activity). Because of differences in the degree of purification, the latter expression is considered to be more suitable for comparison of activities among different enzyme forms. Although all the enzyme forms

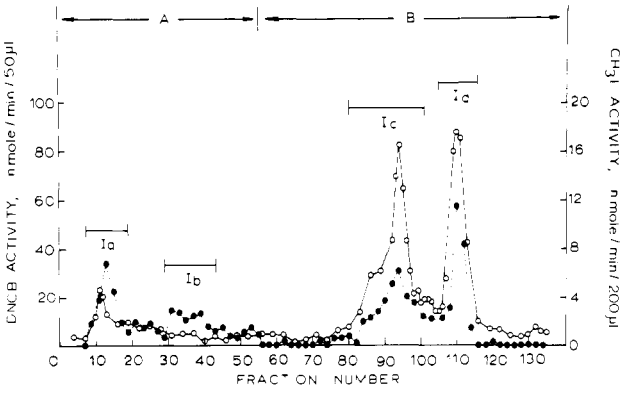

Figure 1. CM-cellulose chromatography of the fraction I enzymes: (A) 250 mL of 10 mM potassium phosphate, pH 6.7; (B) a linear gradient between 150 mL of the buffer and 150 mL of the buffer containing 75 mM KCl; DNCB activity (—o—), methyl iodide activity (—●—).

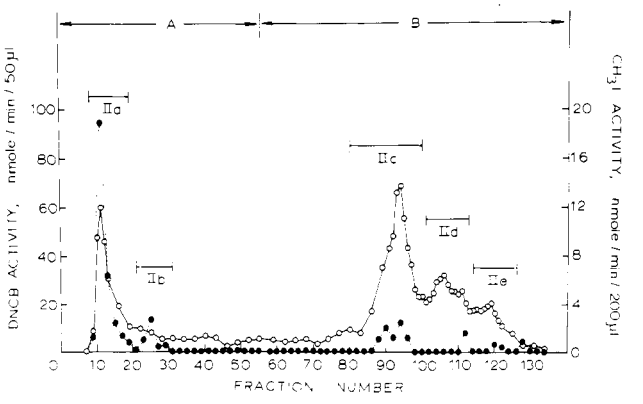

Figure 2. CM-cellulose chromatography of the fraction II enzymes: (A) 250 mL of the 10 mM potassium phosphate, pH 6.7; (B) a linear gradient between 150 mL of the buffer and 150 mL of the buffer containing 75 mM KCl; DNCB activity (—o—), methyl iodide activity (—●—).

were active for this substrate, Ic and Id were particularly active. The results are in contrast to the activities for DNCB (Table I). With DNCB, IIc was the major fraction, and IIa was as active as Ic and Id. Similarly, results with DCNB are presented in Table II. A striking difference was obtained with this substrate between fraction I enzymes and fraction II enzymes. All the enzyme forms derived from fraction I (which were not bound to the DEAE-cellulose column) showed no activity toward this substrate. On the other hand, all the enzyme forms derived from fraction II (which was bound to DEAE-cellulose column) were active toward DCNB. The IIc peak was the most active, followed by IId. Peaks IIa and IIe demonstrated

Table II. Activity toward Methyl Iodide and DCNB of Multiple Forms of Rat Liver Glutathione S-Transferases[a]

enzyme[b] form	methyl iodide		DCNB	
	sp act., nmol min^{-1} mg^{-1}	total act., nmol/min	specific act., nmol min^{-1} mg^{-1}	total act., nmol/min
Ia_1	112.0 ± 36.1	65.1 ± 21.0	0	0
Ia_2	32.8 ± 19.7	21.8 ± 13.1	0	0
Ib	114.6 ± 16.3	41.7 ± 5.9	0	0
Ic	179.8 ± 0.0	243.2 ± 0.0	0	0
Id	225.3 ± 7.2	781.5 ± 25.0	0	0
IIa	9.3 ± 0.8	193.3 ± 16.9	41.5 ± 1.2	866.9 ± 24.5
IIb	41.1 ± 6.4	64.8 ± 10.0	246.2 ± 22.5	387.6 ± 35.4
IIc	52.1 ± 3.5	211.1 ± 14.3	757.0 ± 62.4	3068.2 ± 253.0
IId	65.9 ± 2.6	146.0 ± 5.7	805.9 ± 17.8	1784.3 ± 39.4
IIe	70.4 ± 3.3	66.8 ± 3.2	775.4 ± 46.4	736.2 ± 44.1

[a] The results are the mean of three replications ± SD. [b] All enzyme preparations are from step 5 except for Ib and IIb which are from step 4 (see Table I).

Table III. Activity toward Diazinon of Multiple Forms of Rat Liver Glutathione S-Transferases[a]

enzyme[b] form	sp act., nmol h^{-1} mg^{-1}	total act., nmol/h	% reaction GS-ethyl	% reaction GS-pyrimidinyl
Ia_1	65.1 ± 9.3	37.8 ± 5.4	18	82
Ia_2	56.0 ± 7.7	37.2 ± 5.1	14	86
Ib	100.6 ± 5.4	36.6 ± 2.0	10	90
Ic	134.8 ± 18.6	557.4 ± 83.1	12	88
Id	112.5 ± 4.1	390.2 ± 14.4	11	89
IIa	25.1 ± 0.2	524.7 ± 3.1	5	95
IIb	32.1 ± 0.7	50.5 ± 1.1	6	94
IIc	54.2 ± 2.0	219.5 ± 7.9	9	91
IId	15.5 ± 2.3	34.2 ± 5.2	36	64
IIe	13.4 ± 3.5	12.8 ± 3.3	39	61

[a] The results are the mean of three replications ± SD. [b] All enzyme preparations are from step 5 except for Ib and IIb which are from step 4 (see Table I).

Table IV. Activity toward Methyl Parathion of Multiple Forms of Rat Liver Glutathione S-Transferases[a]

enzyme[b] form	sp act., nmol h^{-1} mg^{-1}	total act., nmol/h	% reaction GS-methyl	% reaction GS-p-nitrophenyl
Ia_1	363.5 ± 7.3	211.2 ± 4.3	>99	<1
Ia_2	312.2 ± 16.5	207.1 ± 11.0	98	2
Ib	634.4 ± 5.2	230.7 ± 1.9	98	2
Ic	655.1 ± 4.3	2708.5 ± 17.7	>99	<1
Id	510.0 ± 1.9	1768.9 ± 6.6	99	1
IIa	46.6 ± 0.5	973.2 ± 9.5	73	27
IIb	109.9 ± 2.1	173.1 ± 3.4	63	37
IIc	100.5 ± 1.8	407.2 ± 7.2	54	46
IId	24.4 ± 1.7	54.1 ± 3.8	67	33
IIe	14.8 ± 6.1	14.0 ± 5.8	78	22

[a] The results are the mean of three replications ± SD. [b] All enzyme preparations are from step 5 except for Ib and IIb which are from step 4 (see Table I).

approximately equal activity for DCNB, in contrast to the findings with DNCB for which IIc was much more active than the IIe (Table I).

Activity toward Organophosphorus Insecticides. Diazinon, parathion, and their analogues are organophosphorus insecticides which are conjugated by two types of glutathione S-transferases reaction (Yang, 1976). Previous studies with housefly enzyme (Motoyama and Dauterman, 1977) demonstrated that the GSH conjugation of diazinon was approximately 63% via O-dealkylation and 37% via aryl group conjugation. With parathion, the reaction percentage was opposite and 28% was via O-dealkylation and 72% was via aryl group conjugation. However, when the ethoxy groups were replaced by methoxy groups, a remarkable increase in overall activity was observed, and the reaction was exclusively via O-dealkylation. Therefore, it was of particular interest to study the reaction of these insecticides with the multiple forms of rat liver glutathione S-transferases.

The results with diazinon are summarized in Table III. All the enzymes were capable of conjugating this substrate, Ic, Id, and IIa being the most active. The IIc enzyme which showed the highest activity for DNCB as well as for DCNB and is considered to be the most active for aryl transfer but did not have the highest activity to diazinon. Separation of the reaction products on TLC indicated that both types of reaction took place. In contrast to the results with the housefly enzymes as mentioned previously, 90% of the reaction by the rat liver enzymes was via the aryl group conjugation. With IId and IIe, the aryl group conjugation accounted for approximately 60%.

Results with methyl parathion are presented in Table IV. Peaks Ic and Id were the predominant ones for this substrate. This is in agreement with their activities for methyl iodide. The separation of the reaction products on TLC revealed four radioactive peaks (Figure 3). Cochromatography with known standards suggested the four radioactive peaks to be GS-p-nitrophenyl, desmethyl parathion, p-nitrophenol, and an unknown. However, further investigations indicated that p-nitrophenol and the unknown were formed nonenzymatically from desmethyl parathion. This finding is based on the results of TLC with sequential developments. When the plate was developed with hexane–ethyl acetate–benzene (4:2:1, v/v) first, all the water-soluble products remained at the origin and the chromatogram showed no existence of p-nitrophenol (Figures 3 and 4). However, the subsequent development of the same plate with the second solvent system, acetonitrile–water (85:15, v/v) (Figure 3B) and then with the first solvent system again (Figure 3C) resulted in the separation of p-nitrophenol, suggesting that the compound was formed nonenzymatically on the silica gel plate with the solvent system containing water. The unknown was not detected when the reaction mixture was applied to the TLC immediately after extraction with chloroform (Figure 3C). However, increasing amounts of the unknown formed with time after extraction (Figure 3D), indicating the compound was formed nonenzymatically during the storage of the reaction mixture. Additional evidence to support the above conjecture is that the amount of GS-p-nitrophenol and the total amount of the four products was always constant, while the amount of the desmethyl parathion, p-nitrophenol, and the unknown varied. Although the identity of the unknown remains to be identified, the compound might be p-nitrophenylphosphorothioic acid produced by further dealkylation of desmethyl parathion.

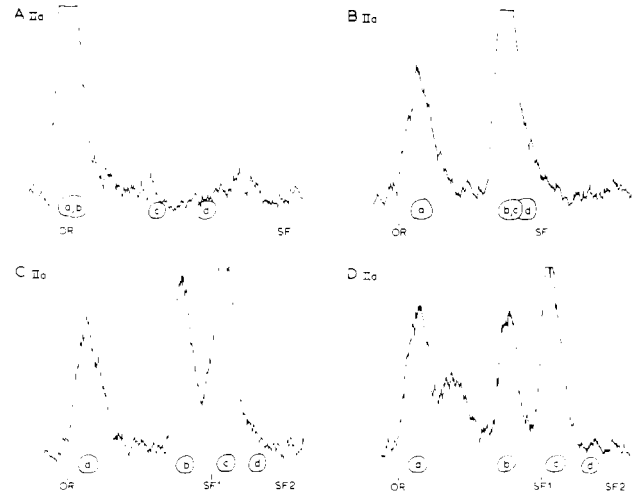

Figure 3. The radiochromatogram of water-soluble products of methyl parathion produced by the IIa form of rat liver glutathione S-transferases. The products were separated by TLC on silica gel N-HR plates with the following solvent developments: (A) hexane–ethyl acetate–benzene (4:2:1 v/v); (B) acetonitrile–water (85:15 v/v); (C) (SF1) with acetonitrile–water (85:15 v/v), (SF2) with hexane–ethyl acetate–benzene (4:2:1 v/v); (D) the same as C, except the aqueous layer was applied after storage in a cold room overnight. Nonradioactive standards cochromatographed were (a) GS-p-nitrophenyl, (b) desmethyl parathion, (c) p-nitrophenol, (d) methyl parathion.

When the amounts of methyl glutathione and p-nitrophenylglutathione were compared, there was a striking difference between fraction I and fraction II enzmes (Table IV). Fraction I enzymes catalyzed the reaction with methyl

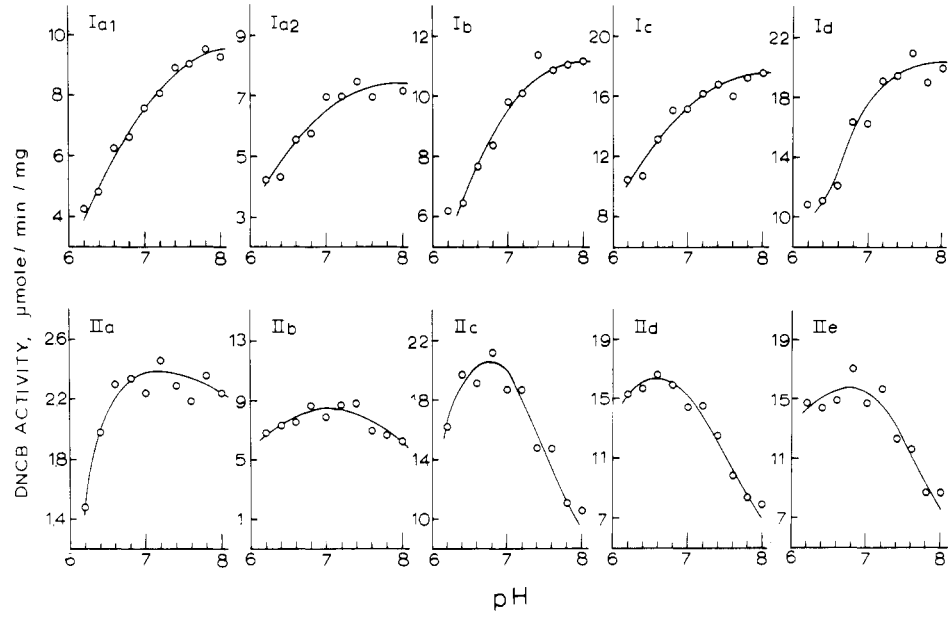

Figure 4. The pH–activity curves of multiple forms of rat liver glutathione *S*-transferases. Enzyme preparations are from step 5 except for Ib and IIb which are from step 4 (see Table I).

parathion entirely via *O*-alkyl conjugation similarly to the housefly enzmes (Motoyama and Dauterman, 1977). In conrast, the fraction II enzymes catalyzed a fairly large part, ranging from 22 to 46%, of the reaction via *p*-nitrophenyl conjugation. Particularly with the enzyme IIc, the reaction ratio between the *O*-alkyl and the leaving group conjugation was almost equal.

Effect of pH on DNCB Activity. Effect of pH on glutathione *S*-transferase activity can be another criteria to compare the identity of multiple enzyme forms obtained. It appears that the optimum pH for glutathione *S*-transferases may vary not only according to the enzyme forms but also with the substrate used. DNCB was chosen in the present study because all the enzymes exhibited activity toward this substrate and it was possible, therefore, to compare the effect of pH for the same substrate for all the enzyme forms. Results are presented in Figure 4. From the pH–activity curves, the difference between the fraction I enzymes showed increased activity at higher pH, leveling off at pH 8.0 which was the highest pH evaluated. In contrast, most of the fraction II enzymes showed a rapid decrease in activity after pH 7.0, except for IIa and IIb for which the decrease in activity was not as sharp. No attempt was made to study the effect of pH greater than 8.0 because of the marked increase in nonenzymatic conjugation with this substrate.

Identity of the Enzymes. Although it is difficult to identify the multiple forms obtained in the present study with those previously reported for rat liver, it seems possible that glutathione *S*-transferases A and C described by Habig et al. (1974b) are similar to the fraction II enzymes. This judgement is based upon the substrate specificity, i.e., these enzymes are highly active for DCNB but have much lower activity for methyl iodide. The enzymes designated as I and II by Askelöf et al. (1975) also seem to correspond to this group. On the other hand, glutathione *S*-transferase B and E (Fjellstedt et al., 1973; Habig et al., 1974a,b) seem to be similar to the fraction I enzymes in the present study. These enzymes were highly active for methyl iodide but had little or no activity for DCNB.

The rat liver enzyme which Hollingworth et al. (1973) studied with paraoxon as the substrate and referred to a glutathione *S*-aryltransferase seems to correspond to the fraction II enzymes. The enzyme catalyzed the reaction almost exclusively via the aryl group conjugation and was found mainly in the liver. On the other hand, when methyl paraoxon was used as the substrate, the reaction was almost exclusively via *O*-alkyl conjugation and the activity was present in both liver and kidney preparations. These authors suggested that the latter reaction was catalyzed by a separate enzyme, glutathione *S*-alkyltransferase. This enzyme seems to be analogous to the fraction I enzymes of the present study.

Usui et al. (1977b) recently separated five forms of glutathione *S*-transferases from rat liver by hydroxylapatite chromatography. Each form showed a different substrate specificity pattern for two organophosphorus insecticides, methyl parathion and diazinon, as well as two noninsecticidal substrates DCNB and methyl iodide. Judging from the pattern, enzymes II, IV, and V appear to correspond to fraction II enzymes of the present paper. A difference was noted where enzymes II and V were active only for DCNB, while all the fraction II enzymes in the present study were active for all of the substrates. Similarly, enzymes I and III seem to correspond to the fraction I enzymes of the present paper. Again, some differences were observed. First, enzyme I had little activity for diazinon, while all the fraction I enzymes in the present paper were active toward this substrate. Second, enzyme III catalyzed both GS-*p*-nitrophenyl and GS-methyl conjugation with methyl parathion, while fraction I enzymes in the present study catalyzed the reaction with this substrate almost exclusively via *O*-alkyl conjugation.

As mentioned earlier, the present study showed that desmethyl parathion can be nonenzymatically hydrolyzed to yield *p*-nitrophenol and monomethylphosphorothioic acid on the silica gel plate. In the present study, ^{14}C-*p*-nitrophenyl-labeled methyl parathion was used, and the product detected was *p*-nitro[^{14}C]phenol (Figure 3). However, Usui et al. (1977b) used ^{14}C-methoxy labeled substrates in their studies, and the product detected for nonenzymatic hydrolysis of desmethyl parathion would have been monomethylphosphorothioic acid. An attempt in our laboratory to separate dimethyl phosphorothioic acid and monomethyl phosphorothioic acid using their TLC system resulted in overlapping R_f values for both

compounds. Therefore, the radioactivity which was detected as dimethylphosphorothioic acid could have been partially derived from monomethylphosphorothioic acid.

In comparison with the five forms of glutathione S-transferases separated from fat body of the American cockroach (Usui et al., 1977a), the pH–activity curves indicated that enzymes I and V are similar to the fraction II enzymes, and enzymes II, III, and IV correspond to the fraction I enzymes of the present study. However, since all their enzymes were active for DCNB, it appears that the cockroach enzymes are somewhat different from the rat liver enzymes. They appear to be more similar to the housefly enzyme which had a rather high optimum pH and was active for methyl parathion, diazinon, methyl iodide, and DCNB (Motoyama and Dauterman, 1977).

Although it appears certain that the rat liver glutathione S-transferases can be classified into at least two major groups, the identity of multiple forms further separated on CM–cellulose or hydroxylapatite columns may require further study. Recent studies on molecular weight, subunits, and multiple forms of housefly glutathione S-transferase demonstrated that multiple forms can be derived from the same enzyme on ion-exchange columns or on electrophoresis by dissociation, aggregation, or by binding with artifacts (Moyoyama and Dauterman, 1978). One of the dissociated forms of housefly enzyme and the native form showed differences in certin properties, i.e., molecular weight, molecular charge, specific activity, and activation energy. Studies of mammalian liver enzymes also indicated that the dissociation or interconversion of multiple forms of glutathione S-transferases occurred (Gillham, 1973; Fjellstedt et al., 1973; Kamisaka et al., 1975).

In conclusion, despite the differences mentioned above, the substrate specificities of the multiple forms of the rat liver enzymes in the present study were in general agreement with the findings of the previous workers (Shishido et al., 1972; Hollingworth et al., 1973; Usui et al., 1977b). Rat liver glutathione S-transferases can be divided into two major groups: (1) fraction I enzymes which do not bind to a DEAE–cellulose column, precipitate with a low ammonium sulfate saturation, have a high optimum pH, and a substrate preference toward alkyl groups of insecticidal as well as noninsecticidal compounds; (2) fraction II enzymes which bind to a DEAE–cellulose column, require a higher ammonium sulfate saturation for precipitation, have a lower optimum pH, and a substrate preference toward aryl groups of insecticidal as well as noninsecticidal compounds. Certain organophosphorus insecticides can be conjugated by glutathione S-transferases at the O-alkyl and/or the leaving groups. The amount of conjugation of the alkyl or aryl group depends upon both the structure of the insecticides as well as the forms of the enzymes.

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