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Purification and Characterization of Three Distinct Glutathione Transferases from Mouse Liver†

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Received February 26, 1985; Revised Manuscript Received February 13, 1986

ABSTRACT: Three distinct glutathione transferases in the liver cytosol fraction of male NMRI mice have been purified by affinity chromatography and fast protein liquid chromatofocusing. These enzymes account for approximately 95% of the activity detectable with 1-chloro-2,4-dinitrobenzene as electrophilic substrate. Differences between the three forms are manifested in isoelectric points, apparent subunit molecular mass values, amino acid compositions, N-terminal structures, substrate specificities, and sensitivities to inhibitors, as well as in reactions with specific antibodies raised against glutathione transferases from rat and human tissues. The results indicate strongly that the three mouse enzymes are products of different genes. A comparison of the mouse glutathione transferases with rat and human enzymes revealed similarities between the transferases from different species. Mouse glutathione transferases have been named on the basis of their respective subunit compositions.

The glutathione transferases catalyze the conjugation of glutathione with numerous compounds carrying an electrophilic center (Chasseaud, 1979; Jakoby & Habig, 1980). They are also capable of binding nonsubstrate ligands, such as bilirubin and azo dye carcinogens (Smith & Litwack, 1980).

In many tissues, multiple forms of glutathione transferase are present (Mannervik, 1985). The different enzymes of rat liver (Jakoby & Habig, 1980; Mannervik & Jensson, 1982) and human liver (Kamisaka et al., 1975; Awasthi et al., 1980; Warholm et al., 1980, 1983) have been the subject of various studies, whereas the glutathione transferases in mouse liver have received less attention. In the mouse, strong interstrain

† This work was supported by grants to B.M. from the Swedish Natural Science Research Council and from Harald and Greta Jeanssons Stiftelse.
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0006-2960/86/0425-4119$01.50/0

variation in glutathione transferase activity against several substrates has been observed (Wheldrake et al., 1981), indicating differences in enzyme patterns. Lee et al. (1981) have purified three forms of glutathione transferase from liver of DBA/2J mice. One of these forms is very similar to or identical with one of the two major isoenzymes purified from liver of CD-1 mice (Pearson et al., 1983). We now report the purification of three distinct glutathione transferases from the liver of male NMRI mice. The properties of the enzymes suggest that these three forms of glutathione transferase are products of different genes.

It was recently demonstrated that glutathione transferases from different mammalian species can be grouped into three distinct and species-independent classes named Alpha, Mu, and Pi (Mannervik et al., 1985b). The three transferases purified from mouse liver in this work represent each of the three classes.

Materials and Methods

Chemicals. Benzo[*a*]pyrene 4,5-oxide was obtained from the Cancer Research Program of the National Cancer Institute, Division of Cancer Cause and Prevention, Bethesda, MD. Δ^5-Androstene-3,17-dione was kindly provided by Drs. A. M. Benson and P. Talalay, Johns Hopkins University, Baltimore, MD. 4-Hydroxynonenal and 4-hydroxydecenal were generous gifts from Dr. H. Esterbauer, Institut für Biochemie, Universität Graz, Graz, Austria. All other chemicals were standard commercial products. *S*-Hexylglutathione–Sepharose 6B was prepared as described (Mannervik & Guthenberg, 1981).

Animals. Outbred male NMRI (Naval Medical Research Institute, Bethesda, MD) mice were used.

Enzyme Assays. The glutathione transferase activity was measured with various substrates by methods previously used (Warholm et al., 1981). The conjugation of 4-hydroxyalk-2-enals with glutathione was monitored spectrophotometrically at 224 nm (ϵ = 13 750 M^{-1} cm^{-1}) with 0.1 mM 4-hydroxynon-2-enal (or 4-hydroxydec-2-enal) and 0.5 mM glutathione in 0.1 M sodium phosphate buffer (pH 6.5) (Ålin et al., 1985a). Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Purification of Glutathione Transferases. Mouse livers (34 g from 32 animals) were homogenized in ice-cold 0.25 M sucrose to obtain a 20% (w/v) homogenate. The homogenate was centrifuged at 15000*g* for 20 min. The centrifugation and all subsequent purification steps up to the chromatofocusing were carried out at 4 °C. The supernatant fraction was then centrifuged at 100000*g* for 1 h. The resulting supernatant was passed through a Sephadex G-25 column (9 × 16 cm) packed in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.2 mM dithioerythritol (buffer A) and then applied onto a column of *S*-hexylglutathione–Sepharose 6B (2 × 12 cm) equilibrated with the same Tris buffer. The column was washed with buffer A containing 0.2 M NaCl until no protein could be detected in the effluent. Glutathione transferases were then eluted with 5 mM *S*-hexylglutathione dissolved in buffer A containing 0.2 M NaCl. The active fractions were pooled, and *S*-hexylglutathione was removed on a column of Sephadex G-25 (4 × 29 cm) packed in buffer A. The sample was concentrated to 10 mL in an Amicon ultrafiltration cell with a PM10 membrane.

The different forms of mouse glutathione transferase were separated at 22 °C by fast protein liquid chromatography (FPLC) with the chromatofocusing column Mono P HR 5/20 (Pharmacia Fine Chemicals, Uppsala, Sweden). A sample (9 mL) was adjusted to pH 9 with 25 mM triethylamine, filtered through a 0.22-μm Millipore filter, and applied to the chromatofocusing column. The matrix was equilibrated with 25 mM triethylamine/HCl (pH 9.6). The activity was eluted with buffer made of 1 mL of Pharmalyte (pH 8–10.5) and 5.2 mL of Polybuffer 96, diluted with deionized water, and adjusted to pH 8.0 with 1 M HCl to give a final volume of 300 mL. The flow rate was 1 mL/min. The glutathione transferases eluted in peaks I–III (Figure 1) were further purified individually on the cation exchanger Mono S HR 5/5 of the FPLC system. The Mono S column was equilibrated with 10 mM 4-morpholineethanesulfonic acid (Mes)/NaOH (pH 6.2). The samples were adjusted to pH 6.2 with 10 mM Mes before application to the column. The column was eluted at a flow rate of 2 mL/min with a linear salt gradient, from 0 to 0.5 M NaCl, in the Mes buffer. The purity of each peak was verified by dodecyl sulfate/polyacrylamide gel electrophoresis performed essentially as described by Laemmli (1970).

Table I: Purification of Mouse Liver Glutathione Transferases

fraction	vol (mL)	total act. (μmol/min)[a]	sp act. [μmol·min^{-1}·(mg of protein)$^{-1}$][a]	yield (%)
liver supernatant	127	3800	3.0	100
S-hexylglutathione–Sepharose 6B + Sephadex G-25	70	3300	78	87
Mono P				
peak I	5.3	52	14	1.4
peak II	10	830	71	22
peak III	12	880	150	23
Mono S				
peak I	2.3	37	19	1.0
peak II	3.3	780	119	20
peak III	3.7	570	148	15

[a] Activity measured at 30 °C and pH 6.5 with 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione as substrates.

Antibodies. Antibodies raised in rabbits against rat and human glutathione transferases were available in the laboratory.

Isoelectric Focusing. Isoelectric focusing was performed in a 110-mL column at 4 °C in a sucrose gradient according to instructions by the manufacturer (LKB, Bromma, Sweden). To obtain a pH gradient in the range 7.5–11.5, 1 part of Ampholine pH 7–9 and 14 parts of Ampholine 9–11 were mixed and used as ampholytes. The sample used (9.2 mL) was a Sephadex G-25 pool containing glutathione transferases obtained after the affinity chromatography step in the purification procedure.

Molecular Mass Estimation. The molecular mass for the mouse glutathione transferases was determined by gel filtration on Sephacryl S-200 superfine (Pharmacia, Sweden) with the following standards (molecular mass values within parentheses): lactate dehydrogenase (130 000), glutathione reductase (110 000), malate dehydrogenase (65 000), glyoxalase I (46 000), and cytochrome *c* (13 500).

Results

Enzyme Purification. The results of a typical purification of glutathione transferases from mouse liver cytosol are summarized in Table I. Affinity chromatography on *S*-hexylglutathione–Sepharose 6B resulted in a more than 25-fold purification with little loss of total enzymatic activity. Only a few percent of the enzyme activity applied to the affinity column passed unretained, indicating that no major glutathione

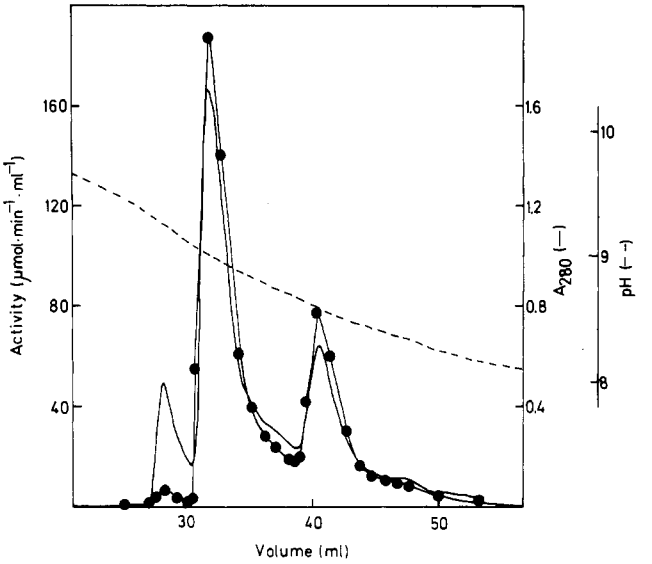

FIGURE 1: Chromatofocusing of mouse glutathione transferases in the FPLC system. The sample (9 mL) was a cytosol fraction purified by affinity chromatography on *S*-hexylglutathione–Sepharose 6B. The enzyme activity (●) was monitored with 1-chloro-2,4-dinitrobenzene as electrophilic substrate, and the three peaks of activity are designated transferase MI, MII, and MIII in the order that they are eluted. The isoelectric points and the apparent subunits molecular mass values were 9.7/25 000, 8.7/23 000, and 8.5/26 500 for transferases MI, MII, and MIII, respectively. A_{280} (—) and pH (– –) were monitored continuously.

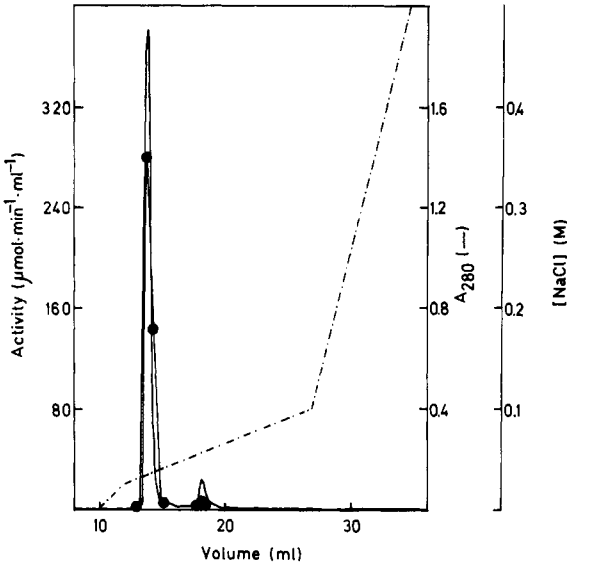

FIGURE 2: Cation exchange chromatography of fraction MII of glutathione transferase from mouse liver. The sample (3 mL) was from the chromatofocusing (Figure 1) and was chromatographed on a Mono S column in the FPLC system. The enzyme activity (●) was measured with 1-chloro-2,4-dinitrobenzene. The small peak eluted at 18 mL consisted of contaminating transferase MI. A_{280} (—) and NaCl concentration (–·–).

transferase isoenzyme, active with 1-chloro-2,4-dinitrobenzene, was lost in this step.

Three forms of glutathione transferase were separated by use of chromatofocusing in the basic pH region in the FPLC system (Figure 1). This system has previously been used in our laboratory to successfully separate the glutathione transferases of different rat tissues (Ålin et al., 1985b; Guthenberg et al., 1985; Robertson et al., 1985). The three enzyme forms were eluted in the order of decreasing isoelectric points and were designated as mouse glutathione transferases MI, MII, and MIII.

The three components eluted from the chromatofocusing column were further purified individually on a column containing a cation exchanger (Figure 2). The resulting preparations of the three isoenzymes were all homogeneous as judged by the appearance of a single band upon analysis by polyacrylamide gel electrophoresis.

Molecular Properties of the Enzymes. Gel filtration experiments under nondissociating conditions gave a relative molecular mass of approximately 45 000 for the three mouse glutathione transferases. The apparent molecular mass values of the subunits were estimated by dodecyl sulfate/polyacrylamide gel electrophoresis in the presence of rat liver transferase subunits 1, 2, and 3 as molecular mass standards (Jakoby et al., 1984). The results of these experiments indicate that transferases MI, MII, and MIII are homodimers composed of subunits with apparent molecular mass values of 25 000, 23 000, and 26 500, respectively. Amino acid analyses of 24-h hydrolysates of transferases MI, MII, and MIII demonstrated clear differences among the enzymes (Table II). Determinations of the tryptophan content of the proteins were not included. Isoelectric focusing at 4 °C in a pH gradient in the range 7.5–10.5 gave isoelectric points of 9.7, 8.7, and 8.5 for MI, MII, and MIII, respectively (Figure 3).

Immunological Properties. Ouchterlony double-diffusion immunoprecipitation experiments were performed in order to explore possible structural relationships among the three mouse

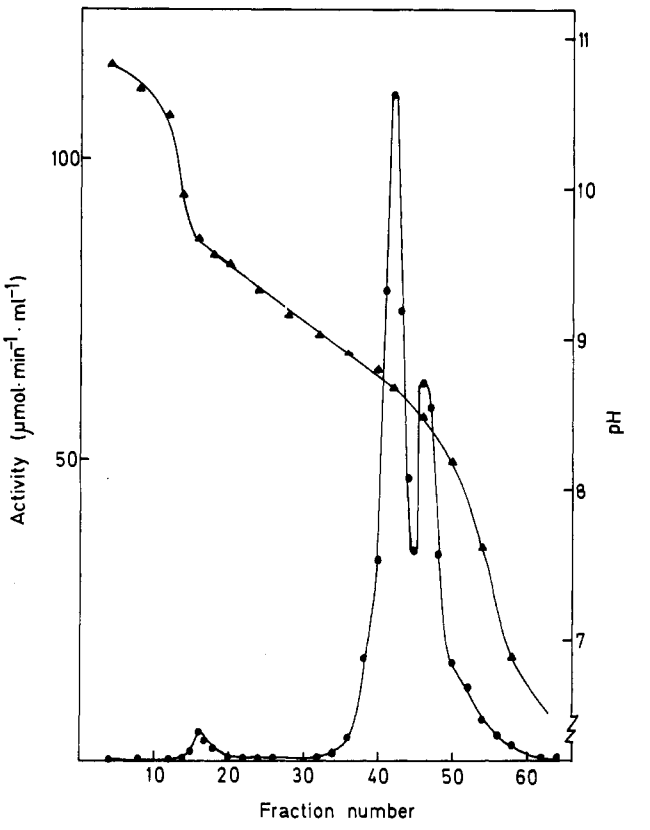

FIGURE 3: Isoelectric focusing of mouse glutathione transferases. The sample was a cytosol fraction purified by affinity chromatography on *S*-hexylglutathione–Sepharose 6B. Enzyme activity (●) was measured with 1-chloro-2,4-dinitrobenzene; pH (▲) was measured at 4 °C.

transferases as well as between the mouse enzymes and transferases from rat and man. Figure 4 shows some of the combinations tried. Antibodies raised against rat glutathione transferases 1-1, 2-2, 3-3, 4-4, and 7-7 and human transferases α–ϵ, μ, and π were used. Mouse transferase MI gave a precipitin line (partial identity as indicated by spur formation)

Table II: Amino Acid Analyses of Mouse Liver Glutathione Transferases[a]

amino acid	transferase MI	MII	MIII
Cys	1.0	3.1	2.4
Asx	26.9	29.5	30.2
Thr	5.5	8.9	9.8
Ser	12.4	10.1	12.2
Glx	24.4	24.3	23.6
Pro	14.2	17.5	15.2
Gly	15.2	21.2	12.9
Ala	18.2	14.5	12.8
Val	15.4	11.2	5.9
Met	6.6	4.8	7.0
Ile	11.5	10.1	13.8
Leu	31.0	33.6	27.4
Tyr	10.9	12.9	14.2
Phe	10.8	8.8	14.1
Lys	18.6	13.1	18.8
His	4.5	4.3	5.2
Arg	13.2	11.6	15.2

[a] The values are molar ratios determined after 24-h hydrolysis at 110 °C with 6 M HCl and 0.5% phenol. The calculations are based on a subunit M_r of 26 000 for all subunits.

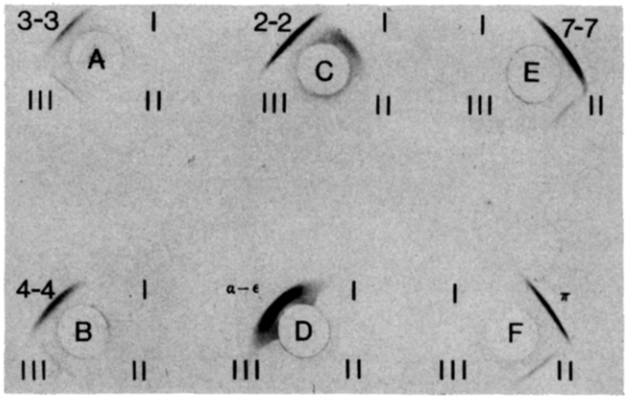

FIGURE 4: Ouchterlony double-diffusion immunoprecipitation analysis of mouse glutathione transferases. Center wells contained antibodies against human or rat glutathione transferases as indicated: antibodies against transferase rat 3-3 (A), rat 4-4 (B), rat 2-2 (C), human α–ϵ (D), rat 7-7 (E), and human π (F). Peripheral wells around the center wells contained the antigen used to raise the antibodies as well as the different mouse transferases (Roman numerals). The precipitates were stained with Coomassie Brilliant Blue R-250.

with antibodies against rat transferase 2-2 but not against any of the other antibodies. Mouse transferase MII reacted with antibodies against rat transferase 7-7 and human transferase π (partial identity). Mouse transferase MIII gave a precipitate of apparent identity with antibodies to rat transferase 3-3 and a precipitate of partial identity with anti-transferase 4-4 antibodies. No further precipitates were detected with any other combination of the mouse transferases and the eight different antibody preparations.

Substrate Specificities. The substrate specificities of the purified mouse liver glutathione transferases are shown in Table III. Transferase MI has a high glutathione peroxidase activity, as shown by the specific activity with cumene hydroperoxide. Ethacrynic acid is a good substrate for transferase MII, whereas transferase MIII contains comparatively high activities with 1,2-dichloro-4-nitrobenzene and bromosulfophthalein. The best substrate for all isoenzymes is 1-chloro-2,4-dinitrobenzene. The mouse liver transferases also show some, but not very high, steroid isomerase activity. Interestingly, this isomerase activity is inhibited by reduced glutathione. This inhibitory effect is prominent for the isomerase activity of transferase MII, which is inhibited by 60%

Table III: Specific Activities of Mouse Liver Glutathione Transferases with Various Substrates

substrate	sp act. (μmol·min^{-1}·mg^{-1}) for transferase MI	MII	MIII
1-chloro-2,4-dinitrobenzene	19	119	148
1,2-dichloro-4-nitrobenzene	0.062	0.14	4.4
bromosulfophthalein	0.008	0.007	0.58
ethacrynic acid	0.025	1.4	0.12
trans-4-phenyl-3-buten-2-one	0.009	0.013	0.044
1,2-epoxy-3-(*p*-nitrophenoxy)propane	0.23	0.77	0.48
benzo[*a*]pyrene 4,5-oxide	0.009	0.033	0.076
cumene hydroperoxide	11.6	0.14	0.11
H_2O_2	<0.03	<0.03	<0.03
Δ^5-androstene-3,17-dione[a]	0.035	0.14	0.043
p-nitrophenyl acetate	0.011	0.21	0.59
4-hydroxynonenal	1.1	2.6	6.0
4-hydroxydecenal	1.3	3.0	4.6

[a] The activity is measured in the absence of reduced glutathione since this compound inhibits the isomerase activity of the mouse glutathione transferases.

Table IV: Inhibition Parameters, I_{50} Values (μM),[a] for Mouse Liver Glutathione Transferases

inhibitor	I_{50} (μM) of transferase MI	MII	MIII
bromosulfophthalein	100	70	7
Cibacron Blue	10	0.1	0.7
hematin	0.5	4	2
S-hexylglutathione	7	10	7
S-(*p*-bromobenzyl)glutathione	10	5	15
tributyltin acetate	0.1	4	0.07
triethyltin bromide	4	5	0.1
triphenyltin chloride	0.3	10	0.04
Rose Bengal	15	5	0.7
indomethacin	>200	>200	200

[a] The glutathione transferase activity was measured at pH 6.5 with 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione as substrates.

in the presence of 0.1 mM glutathione. In contrast, the isomerase activity of the glutathione transferases from rat and human liver is strictly dependent upon the presence of glutathione.

Sensitivity to Various Inhibitors. The inhibitory effects of a number of substances on the enzymatic activity of the three different mouse liver transferases are shown in Table IV. It was found that large differences exist in the sensitivities of the enzymes. The differences can be used for distinction of the three forms of glutathione transferase. For example, transferase MIII is almost 10 times more sensitive to triphenyltin chloride than transferase MI, which, in turn, is approximately 30 times more sensitive than transferase MII to this inhibitor. Indomethacin was included as an inhibitor, since it has previously been found to selectively inhibit rat transferase 4-4 (Nicholls & Ahokas, 1984; Ålin et al., 1985b).

Figure 5 shows a plot of enzyme activity vs. triphenyltin chloride concentration for transferase MIII. The symmetrical shape of the curve and the maximal negative slope of 0.58 at 50% inhibition are properties typical for a homodimeric enzyme with noncooperative subunits, earlier found for homodimeric rat transferases (Tahir & Mannervik, 1986). By these criteria, the inhibition experiments were consistent with the view that each of the three mouse enzymes is composed of two identical subunits.

Discussion

Three distinctly different forms of glutathione transferase have been purified from livers of outbred male NMRI mice. The purification procedure includes affinity chromatography

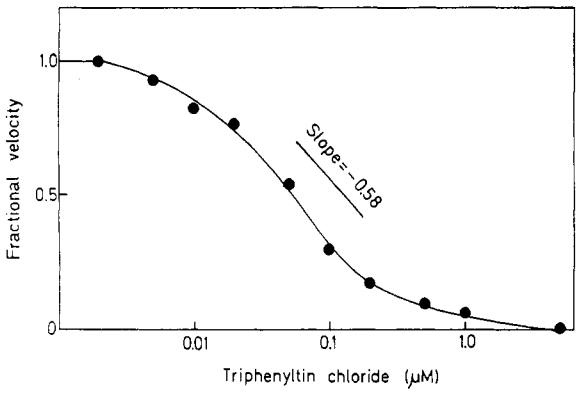

FIGURE 5: Inhibition of mouse glutathione transferase MIII with triphenyltin chloride. Fractional velocity was determined by comparison with enzyme in the absence of inhibitor. Assays were made at pH 6.5 with 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione as substrates. The straight line indicates the slope [(−ln 10)/4] expected at 50% inhibition for linear inhibition of a homodimeric (noncooperative) transferase [cf. Tahir & Mannervik (1986)].

on *S*-hexylglutathione–Sepharose 6B (Guthenberg & Mannervik, 1979; Warholm et al., 1981), which gives extensive purification in combination with high yield. All well-characterized cytosolic forms of glutathione transferase, except rat transferase 5-5 (Meyer et al., 1984), are retained on this affinity column. The FPLC system for chromatofocusing and for the subsequent ion-exchange chromatography allows rapid separation of the multiple forms of glutathione transferase. The entire purification procedure can be completed in 2 days.

The three enzymes purified are the predominant cytosolic glutathione transferases in the liver of the male NMRI mouse. Figure 1 shows that in terms of protein the relative abundance of the three isoenzymes decreases in the order MII > MIII > MI, even though the values are of the same order of magnitude. Analysis of the activity profile after isoelectric focusing of a liver supernatant fraction revealed minor amounts (<5%) of additional, more acidic, enzyme(s) (measured with 1-chloro-2,4-dinitrobenzene, data not shown).

Glutathione transferases MI, MII, and MIII all appear to be dimers of identical subunits, in contrast to the basic hepatic isoenzymes in the rat, which include heterodimeric structures in addition to homodimers (Mannervik & Jensson, 1982). Inhibition curves (cf. Figure 5) were examined for deviations from a simple linear inhibition as an indication for heterodimeric enzymes [cf. Tahir & Mannervik (1986)], but all data were consistent with homodimeric structures for the mouse enzymes investigated. The N-terminal structures determined for transferases MII and MIII (Mannervik et al., 1985b) also support the conclusion that the enzymes are homodimeric, since unique sequences were obtained. In the case of a heterodimeric rat transferase, two amino acids were obtained for certain positions in the N-terminal sequence analyzed (Frey et al., 1983).

Results of amino acid analyses strongly suggest that the isoenzymes are encoded in different genes. Val is most abundant in transferase MI, Gly in MII, and Phe in MIII (Table II), and since a product cannot contain more of an amino acid than the parent molecule, these findings preclude precursor–product relationships in which one isoenzyme by partial hydrolysis gives rise to another. Analogous conclusions about three different isoenzymes of human glutathione transferase have previously been reached (Warholm et al., 1983; Ålin et al., 1985c).

Antibodies have not yet been raised against mouse transferases MI, MII, or MIII, but specific antibodies to five rat and three human transferases were used to define structural differences among the mouse enzymes. Each of the mouse transferases gave a positive precipitin reaction with at least one antiserum, but in no case did two mouse isoenzymes give a precipitate with the same antiserum. This finding indicates that some isoenzymes from different species contain an identical epitope and that this epitope is not common to all the multiple forms of glutathione transferase in a single species.

The apparent molecular mass values for the subunits determined by dodecyl sulfate/polyacrylamide gel electrophoresis are similar to values obtained for subunits of certain rat enzymes (Jakoby et al., 1984; Guthenberg et al., 1985); the following pairs have similar mobilities: subunit MI and rat subunit 1; subunit MII and rat subunit 7; subunit MIII and rat subunit 3. The values serve to characterize the different subunits, even though studies of rat cDNA sequences have shown that the true molecular mass values differ less than indicated by electrophoresis (Pickett et al., 1985).

The studies of the substrate specificities and the sensitivities to inhibitors (Tables III and IV) provide additional properties that distinguish the three types of glutathione transferase isolated from mouse liver. Transferase MI carries most of the non-selenium-dependent glutathione peroxidase activity; as in other animal species, none of the glutathione transferase isoenzymes has significant activity with H_2O_2 (Wendel, 1980). Transferase MII has the highest activity with ethacrynic acid, which is a representative of alkene substrates, whereas transferase MIII has the highest activity with the classical aryl transferase substrates 1,2-dichloro-4-nitrobenzene and bromosulfophthalein (Boyland & Chasseaud, 1969). Thus, multiple criteria exist for future identification of the above enzymes in other tissues or in other strains of mice.

The "natural" substrates for the glutathione transferases are unknown, but products of oxidative metabolism have been suggested as likely candidates (Mannervik et al., 1985a). Thus, 4-hydroxyalkenals, which are products of lipid peroxidation, are among the substrates giving the highest specific activities [Table III; cf. Ålin et al. (1985a)]. Also, epoxides and organic hydroperoxides should be considered as substrates arising in metabolism. However, so far only the activity with organic hydroperoxides suggests a specific biological function that is characteristic for one (transferase MI) of the three distinct isoenzymes.

Two isoenzymes of glutathione transferase previously isolated from mouse liver, called GT-8.7 and GT-9.3 (Pearson et al., 1983), can be compared with transferases MI, MII, and MIII studied in this work. Transferase MIII is closely related to GT-8.7 by the criteria of isoelectric point, apparent subunit molecular mass, and high relative activity with 1,2-dichloro-4-nitrobenzene. Further, N-terminal amino acid sequence analysis of transferase MIII (Mannervik et al., 1985b) gave results for the first 20 residues that are identical with the corresponding residues reported for GT-8.7 (Pearson et al., 1983). Transferases MI and MII were found to have different N-termini; none of them was similar to that of GT-8.7 or GT-9.3 (or MIII). The second of the two isoenzymes characterized in structural detail by Pearson et al. (1983) has not been found in this investigation. One explanation may be that GT-9.3 is reported as a minor isoenzyme (Pearson et al., 1983). Another cause may be that different strains (CD-1 vs. NMRI) and different sexes were used. Differences between sexes in the occurrence of glutathione transferases in rat liver have been reported (Igarashi et al., 1985).

Another set of hepatic glutathione transferases was isolated from male mice of the DBA/2J strain (Lee et al., 1981). Three isoenzymes, denoted F1, F2, and F3, were obtained in

Table V: Comparison of Amino Acid Compositions of Mouse Glutathione Transferases and Enzymes from Other Species Using the Difference Index of Metzger et al. (1968)

glutathione transferase	difference index calculated for transferase[a]		
	MI	MII	MIII
mouse MI			
mouse MII	8.9		
mouse MIII	8.8	10.6	
mouse F2[b]	6.5	4.8	9.9
mouse F3[b]	9.6	11.7	4.9
rat 1-1[c]	8.4	13.5	11.7
rat 2-2[c]	5.2	11.8	11.9
rat 3-3[c]	9.1	12.7	4.4
rat 4-4[c]	7.7	9.8	5.7
human α–ϵ[d]	8.2	13.9	12.4
human μ[d]	8.3	11.4	6.7
human π[d]	10.2	8.0	16.1

[a] Values for Cys and Trp are not included in the calculations of the indices. The amino acid compositions for mouse transferases MI, MII, and MIII are from Table II. [b] From Lee et al. (1981). [c] Unpublished work of P. Ålin, H. Jörnvall, and B. Mannervik [cf. Mannervik (1985)]. [d] From Warholm et al. (1983).

pure form. Isoenzymes F1 and F2 were very similar, whereas the third isoenzyme, F3, was distinguished from F1 and F2 by amino acid composition, tryptic peptide pattern, apparent subunit molecular mass, isoelectric point, and high relative activity with 1,2-dichloro-4-nitrobenzene. By the same criteria, F3 can with high confidence be related to GT-8.7 or transferase MIII. Forms F1 and F2 appear similar to transferase MII, judging from apparent subunit molecular mass and amino acid composition. However, lack of amino acid sequence data or a more extensive enzymatic characterization of these forms prevents a detailed comparison with transferase MII.

The relationship between different forms of glutathione transferase was also evaluated by comparison of their respective amino acid compositions with the difference index of Metzger et al. (1968). In theory, identical proteins should give a value of zero, but the index will not be lower than a certain value due to the experimental error in the data. Thus, mouse transferases MIII and F3, believed to be closely related or identical, give a difference index of 4.9 (Table V). Similarly, transferases MII and F2 give a value of 4.8. Analysis of pairs of isoenzymes from different species indicates that mouse transferase MI is most closely related to rat transferase 2-2 and mouse transferase MIII is most similar to rat transferases 3-3 and 4-4. The examination of similarities between the human and mouse enzymes suggests that human transferases α–ϵ are most closely related to mouse transferase MI, human μ is most closely related to mouse MIII, and human π is most closely related to mouse MII.

The multiple forms of glutathione transferase from different species can be grouped into three distinct classes defined on the basis of enzymatic and structural properties (Mannervik et al., 1984, 1985b). Each of the three mouse enzymes was assigned to one of the three classes: transferase MI to class Alpha, transferase MII to class Pi, and transferase MIII to class Mu (Mannervik et al., 1985b). Thus, it appears that the similarities between the multiple forms of glutathione transferase in different mammalian species are greater than earlier indicated. Studies of the enzyme families in additional species may shed further light on both the molecular evolution and the biological functions of these abundant proteins.

Designations for Multiple Forms of Glutathione Transferase. The glutathione transferases from rat liver cytosol have been named on the basis of their respective subunit compositions. Each variant protein subunit characterized has been designated by an Arabic numeral, and enzymes are identified by their constituent subunits (Jakoby et al., 1984). For example, the heterodimeric enzyme composed of subunits 1 and 2 was named rat glutathione transferase 1-2. In extending the system to the mouse transferases, it was decided that the mouse enzymes should not only be identified by subunit numbers but also be distinguished by a letter signifying the strain from which the protein was isolated,[1] the reason being that preliminary data suggest that homologous transferases from different mouse strains may show significantly different activities with some substrates. Thus, isoenzyme GT-8.7 from the CD-1 strain (Pearson et al., 1983) is called mouse glutathione transferase C 1-1, F3 from the DBA/2J strain (Lee et al., 1981) is called transferase D 1-1, and MIII from the NMRI strain (this investigation) is called transferase N 1-1. Isoenzyme GT-9.3, a minor form (Pearson et al., 1983), is called mouse glutathione transferase C 2-2. Isoenzymes MII and MI (this investigation) are called mouse glutathione transferases N 3-3 and N 4-4, respectively.

ACKNOWLEDGMENTS

We thank the Cancer Research Program of the National Cancer Institute, Bethesda, MD, for providing benzo[*a*]pyrene 4,5-oxide, Drs. A. M. Benson and P. Talalay, Department of Pharmacology, The Johns Hopkins University School of Medicine, Baltimore, MD, for several shipments of Δ^5-androstene-3,17-dione, Dr. H. Esterbauer, Institut für Biochemie, Universität Graz, Graz, Austria, for generous amounts of 4-hydroxyalkenals, and Dr. K. Sato, Second Department of Biochemistry, Hirosaki University School of Medicine, Hirosaki, Japan, for antibodies raised against rat glutathione transferase 7-7. We are also grateful to our colleagues P. Ålin for performing amino acid analyses and K. Larson for drawing the figures of this paper.

Registry No. H_2O_2, 7722-84-1; (*E*)-CH_3COCH=$CHPh$, 1896-62-4; *p*-$O_2NC_6H_4OAc$, 830-03-5; CHOCH=CHCH(OH)$(CH_2)_4CH_3$, 29343-52-0; CHOCH=CHCH(OH)$(CH_2)_5CH_3$, 29389-17-1; Bu_3SnOAc, 56-36-0; Et_3SnBr, 2767-54-6; Ph_3SnCl, 639-58-7; glutathione transferase, 50812-37-8; 1-chloro-2,4-dinitrobenzene, 97-00-7; 1,2-dichloro-4-nitrobenzene, 99-54-7; bromosulfophthalein, 71-67-0; ethacrynic acid, 58-54-8; 1,2-epoxy-3-(*p*-nitrophenoxy)propane, 5255-75-4; benzo[*a*]pyrene 4,5-oxide, 37574-47-3; cumene hydroperoxide, 80-15-9; Δ^5-androstene-3,17-dione, 571-36-8; Cibacron Blue, 53127-08-5; hematin, 15489-90-4; *S*-hexylglutathione, 24425-56-7; *S*-(*p*-bromobenzyl)glutathione, 31702-37-1; Rose Bengal, 11121-48-5; indomethacin, 53-86-1.

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[1] The group appointed for dealing with naming the multiple forms of glutathione transferase (Jakoby et al., 1984) as well as other investigators active in the field (in particular, Dr. A. M. Benson, University of Arkansas, Little Rock, AR) has discussed and agreed upon the designations presented here.

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Biochemical Characterization and Developmental Behavior of *Artemia* Embryonic and Nauplial Deoxyribonucleases†

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Received December 6, 1985; Revised Manuscript Received March 7, 1986

ABSTRACT: Deoxyribonuclease (DNase) activities have been studied during larval development of *Artemia*. The low DNase activity levels detected in extracts of dormant gastrulae were found to increase markedly in the nauplial stages. Partial purification and characterization of this activity in extracts of developed nauplii indicate the presence of three enzymic forms (A-I, A-II, and A-III). These DNase activities of *Artemia* are endonucleases, degrading supercoiled substrates mainly by a single-stranded attack mechanism. They differ in pH profiles, anion sensitivity, reactivity toward NH_2 group reagents, molecular weight, and chromatographic behavior. DNase activity A-I is the only activity detected in extracts of dormant embryos and appears associated with the yolk granules, in a "masked" form. The increase of activity around hatching is mainly due to unmasking of DNase activity A-I during yolk granule metabolism while the further increase during nauplial development correlates with the appearance of DNase A-III.

Artemia is a small crustacean, class *Anostraca*, which has evolved the ecological adaptation of laying dormant gastrulae which can remain in this state for very long periods of time. Upon exposure to the appropriate environmental conditions (Vallejo et al., 1980), development is resumed. After about 20 h, fully formed swimming nauplii hatch from the thick shells which envelop the dormant gastrulae. An interesting feature of this system is that cleavage, i.e., the subdivision of

† This work has been supported by grants from the Rodriguez Pascual Foundation and CAICYT. A.D. was a fellow of the Fondo de Investigaciones Sanitarias.

0006-2960/86/0425-4125$01.50/0