A New Class of Rat Glutathione S-Transferase Yrs-Yrs Inactivating Reactive Sulfate Esters as Metabolites of Carcinogenic Arylmethanols*

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A glutathione (GSH) S-transferase (GST), catalyzing the inactivation of reactive sulfate esters as metabolites of carcinogenic arylmethanols, was isolated from the male Sprague-Dawley rat liver cytosol and purified to homogeneity in 12% yield with a purification factor of 901-fold. The purified GST was a homo-dimeric enzyme protein with subunit M, 26,000 and PI 7.9 and designated as Yrs Yrs because of its enzyme activity toward "reactive sulfate esters." GST Yrs-Yrs could neither be retained on the S-hexylglutathione gel column nor showed any activity toward 1,2-dichloro-4-nitrobenzene, 4-nitrobenzyl chloride, and 1,2-epoxy-3-(4'-nitrophenoxy)propane. 1-Chloro-2,4-dinitrobenzene was a very poor substrate for this GST. 1-Methaphthyl sulfate was the best substrate for GST Yrs-Yrs among the examined mutagenic arylmethyl sulfates. The enzyme had higher activities toward ethacrynic acid and cumene hydroperoxide. N-ternaire amino acid sequence of subunit Yrs, analyzed up to the 25th amino acid, had no homology with any of the known class alpha, mu, and pi enzymes of the Sprague-Dawley rat. Anti-Yrs-IgG raised against GST Yrs-Yrs showed no cross-reactivity with any of subunits Ya, Yc, Yb1, Yb2, and Yp. Anti-IgGs raised against Ya, Yc, Yb1, Yb2, and Yp also showed no cross-reactivity with GST Yrs-Yrs. The purified enzyme proved to differ evidently from the 12 known cytosolic GSTs in various tissues of the rat in all respects. Immunoblot analysis of various tissue cytosols of the male rat indicated that apparent concentrations of the GST Yrs-Yrs protein were in order of liver > testis > adrenal > kidney > lung > brain > skeletal muscle > heart > small intestine = spleen = skin = 0.

In spite of their very weak carcinogenicity, benz[a]anthracene (BA) and chrysene (CR) turn into extremely potent carcinogens on methylation at the L-region (7- and 12-positions) for the former (1-3) and at the 5-position for the latter (4, 5). In hepatic microsomes from untreated rats, enzymatic oxidation of the methylenes takes place more readily at their methyl carbons than at the arene moieties, leading to the formation of reactive arene oxides (6, 7), to yield the corresponding hydroxymethylenes as major metabolites (6-10) which are also potent carcinogens comparable to the mother compounds (1, 2, 5, 11). The carcinogens, 7-hydroxymethyl-BA, 7-hydroxymethyl-12-methyl-BA (7-HMBBA), 12-hydroxy-7-methyl-BA (12-HMBBA), 7,12-dihydroxymethyl-BA (DHBA), and 5-hydroxymethylchrysene (5-HCR) formed from the corresponding methylenes by hepatic microsomal monooxygenases are metabolized further in hepatic cytosol fortified with 3'-phosphoadenosine 5'-phosphosulfate, a cofactor for sulfotransferases, to give highly reactive sulfated esters, Ar-CH₂-OSO₂, except for 5-HCR sulfate with considerable stability (12), all of which have been isolated from incubation mixtures, identified with authentic specimens (12-15), and demonstrated to have potent intrinsic mutagenicity toward Salmonella typhimurium TA 98 (12-15, 17).

As to 7-HMBBA, DHBA, and 5-HCR, the metabolically formed sulfate esters react selectively and readily with the exocyclic amino groups of the purine bases of the cultured DNA through their methylene carbon with loss of a sulfate anion as a leaving group (16, 18, 19). The rat liver cytosolic sulfotransferases that activate these carcinogenic hydroxymethylenes have recently been identified as hydroxysteroid sulfotransferases (16, 20). The same purine base adducts as mentioned above with respects to 7-HMBBA were isolated later by Surh et al. (21) from hepatic DNA of newborn rats given the same carcinogen. However, in adult rats, liver has never been reported to be a target organ for hydroxymethylenes, whereas they show very potent carcinogenicity to the skin of the animals (2).

In the adult rat liver cytosol, the metabolically formed sulfate esters of 5-HCR, 7-HMBBA, and DHBA are enzymatically scavenged so readily by glutathione (GSH) (17, 22, 23) that they cannot exert mutagenicity (17, 22, 23), nor bind covalently to calf thymus DNA (16, 23). From the hepatic cytosolic incubation mixtures fortified with 3'-phosphoadenosine 5'-phosphosulfate and GSH, stable and nonmutagenic S-(aryl)methylglutathiones, Ar-CH₂-SG, have been isolated and identified with authentic specimens (17, 22, 23). These facts, therefore, indicate that GST does play an important role in preventing rat liver, having the highest level of hydroxysteroid sulfotransferase activity among various tissues (24), from tumorigenesis caused by the carcinogens, methyl- and hydroxymethylenes. The present paper deals with (i)

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1 The abbreviations used are: BA, benz[a]anthracene; CR, chrysene; 5-HCR, 5-hydroxymethylchrysene; 7-HMBBA, 7-hydroxymethyl-12-methylbenz[a]anthracene; DIIDA, 7,12-dihydroxymethylbenz[a]anthracene; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; EPNP, 1,2-epoxy-3-(4'-nitrophenoxy)propane; GSH, reduced glutathione; GSSG, oxidized glutathione; S-hexyl-SG, S-hexylglutathione; GST, glutathione S-transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; hplc, high performance liquid chromatography.
the existence of at least three GSTs in the rat liver cytosol, which catalyzed GSH conjugation of reactive sulfates of carcinogenic arylmethanols and were all unretainable on an S-hexy1-SG affinity column, (ii) the isolation and purification of the major one of these GSTs, which was designated as Yrs-Yrs (a homo-dimeric Y protein that catalyzes GSH conjugation of the "reactive sulfate esters") and had no homology in N-terminal amino acid sequence to any of the known classes of rat GST isozymes, (iii) the unique properties of GST Yrs-Yrs in substrate specificity and immunological reactivity, and (iv) the tissue distribution of GST Yrs-Yrs in the rat, estimated by immunoblot analysis.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**Purification and Molecular Significance of Rat Liver Cytosolic GST** for Inactivating Arylmethyl Sulfates—A preliminary study carried out by using rat liver cytosol prior to establishing a purification scheme for GST, tentatively designated as RS, catalyzing the GSH conjugation of 5-HCR sulfate, the most stable compound among highly mutagenic arylmethyl sulfates, provided two important facts; one was that, unlike rat liver soluble GSTs with subunit proteins Ya, Yb1, Yb2, Yc, and Yp, GST RS had little affinity for and, consequently, passed through an S-hexy1-SG labeled Sepharose 6B column, and the other that the enzyme had strong affinity for the sulfonated azo dye-labeled gel (blue Sepharose) column. On direct application of the rat liver cytosol to the S-hexy1-SG-gel column, approximately 96% of the hepatic cytosolic activity for GSH conjugation of 5-HCR sulfate passed through the column. Whereas 86% of the cytosolic activity for GSH conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) was retained at pH 8.25 on this column. Thus, the S-hexy1-SG-labeled affinity column could be used for elimination of the known GST subunit proteins interacting with this column from the rat liver cytosol, and the sulfonated azo dye-labeled column for adsorption of the GST active toward arylmethyl sulfates during the course of purification.

For isolation of GST RS, the rat liver cytosol was applied to an anion-exchange gel (DE-52) column. Most of the cytosolic RS, surveyed by the assay of the 5-HCR sulfate-GSH-conjugating activity, was retained at pH 8.25 on this column and then eluted with NaCl (Fig. 1 in the Miniprint). By this column chromatographic procedure, more than 90% of non-RS proteins were removed. From the RS fraction (Fraction 120-140) of the anion-exchange chromatogram most of non-RS GSTs active toward CDNB were removed by adsorption on the S-hexy1-SG-gel column (Fig. 2 in the Miniprint). The GST RS protein in the flow-through fraction of the S-hexy1-SG-gel column chromatogram was applied to a chromatofocusing gel column to remove non-RS GSTs active toward CDNB completely (Fig. 3 in the Miniprint). The major GST RS protein peak, corresponding to the chromatofocusing fractions (Fraction 54-58) eluted at pH 8.2-8.4, contained no detectable activity toward CDNB (Fig. 3 in the Miniprint). The major GST RS protein isolated by chromatofocusing was collected on and eluted from the blue Sepharose column (Fig. 4 in the Miniprint) and then subjected to gel filtration hplc carried out on a TSK gel G3000 SW column for further purification to homogeneity (Fig. 5 in the Miniprint).

The purified RS preparation, obtained in 12% yield with a purification fold of 901 (Table I), had an apparent Mr value of 54,000, estimated from the retention volume on the TSK gel column in a comparative chromatographic study carried out by using the Mr marker proteins, bovine serum albumin (Mr, 66,000), egg albumin (Mr, 45,000), and bovine carbonic anhydrase (Mr, 29,000). SDS-polyacrylamide gel electrophoresis of the RS preparation indicated that the Mr, 54,000 enzyme protein consisted of two identical subunit proteins with an Mr value of 26,000 (Fig. 6 in the Miniprint), estimated from the result of a comparative electrophoretic study carried out on the same gel plate by using not only various Mr marker proteins but the authentic GST subunit proteins, Ya (Mr, 25,000), Yb1 and Yb2 (Mr, 26,500), and Yc (Mr, 29,000), isolated and purified from the rat liver cytosol. Therefore, based on these molecular data, the homo-dimeric enzyme GST RS was re-designated as GST Yrs-Yrs.

GST Yrs-Yrs had a pI value of 7.9, determined by the isoelectricfocusing method. The N-terminal amino acid sequence of this enzyme, determined from the N-terminal to 25th amino acids with an automatic amino acid sequencer based on the Edman degradation method, was as follows: Gly-Leu-Glu-Leu-Tyr-Leu-Asp-Leu-Leu-Ser-Gln-Pro-Ser-Arg-Ala-Vai-Tyr-Ile-Pha-Ala-Lys-Lys-Asn-Gly-Ile-.

A co-hplc study, carried out on an octadeccisilica column developed with aqueous acetonitrile containing 0.1% (v/v) trifluoracetic acid in gradient manner, indicated that the subunit protein Yrs was eluted as a single peak at a completely different retention time from those of the rat liver cytosolic GST subunits which were collected on and eluted from the S-hexy1-SG affinity column into a single fraction (Fig. 7). Under the hplc conditions, all the dimeric enzymes examined were dissociated into subunits. The subunit Yrs was less polar than the hplc conditions, all the dimeric enzymes examined were dissociated into subunits. The subunit Yrs was less polar than the GST subunits Yb1, Yb2, and Yc and more polar than Ya, the last one of which was eluted as a doublet peak as had been demonstrated by Ostland Farrants et al. (39). These subunit peaks were all identified with the corresponding pure homo-dimeric GSTs isolated from the rat liver cytosol.

Amino acid composition of an acid hydrolyzate of GST Yrs-Yrs indicated that the enzyme contained Glx (Glu and Gln) in the highest molar ratio and, next to this, Leu, Ala, Gly, and Asx (Asp and Asn) in decreasing order (Table II in the Miniprint).

| Purification step | Total protein activity | Specific activity (nmol/min/mg) | Purification fold | Yield % |
|------------------|-----------------------|-------------------------------|------------------|--------|
| Rat liver cytosol | 6248                  | 500                           | 0.08             | 1      |
| DE-52            | 397                   | 390                           | 0.96             | 12     |
| S-Hexyl-SG       | 371                   | 363                           | 0.98             | 12     |
| Chromatofocusing | 20.1                  | 183                           | 9.10             | 114    |
| Blue Sepharose   | 3.94                  | 143                           | 36.3             | 404    |
| TSK gel G3000 SW | 0.86                  | 62                            | 73.1             | 901    |

* Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 1-6, and Table II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
FIG. 7. Separation of rat liver cytosolic GST subunits by reverse phase hplc. A Bondasphere C18-30 Å column (3.9 mm × 15 cm) was eluted at a flow rate of 1 ml/min with a 35-55% (v/v) linear gradient of acetonitrile in water, both containing 0.1% (v/v) trifluoroacetic acid. A, GST fraction (20 μg of protein) isolated from rat liver cytosol by S-hexyl-SG affinity chromatography as described in the text. B, purified GST Yrs-Yrs (5 μg). C, a mixture of A and B in water. U represents unidentified proteins.

was the best substrate for this GST. A kinetic study, carried out by the double reciprocal plot method based on apparent rates for the enzymatic GSH conjugate formation from 5-HCR sulfate (10–50 μM) at pH 7.4, indicated that purified GST Yrs-Yrs had a \( K_m \) value of 17.7 μM and a \( k_{cat} \) value of 0.123 s⁻¹. A \( K_m \) value for GSH was 1.34 mM in the 5-HCR sulfate conjugation reaction catalyzed by this enzyme at pH 7.4.

A comparative study carried out by using purified specimens of six major rat liver cytosolic GSTs with high affinity for the S-hexyl-SG-gel column and high activities toward CDNB indicated that only two GSTs bearing subunit protein Yc could catalyze the GSH conjugation reaction of 5-HCR sulfate (Table IV). However, their activities toward 5-HCR sulfate was only 0.3 and 1% for GSTs Ya-Yc and Yc-Yc of that exerted by GST Yrs-Yrs. Other rat liver GSTs bearing the subunit proteins Yb1 and Yb2 and GST Ya-Ya had no activity toward 5-HCR sulfate, whereas all of these GSTs had high activities toward CDNB that was an extremely poor substrate for GST Yrs-Yrs.

Representative GST substrates were examined for GSH conjugations by GST Yrs-Yrs (Table V). None of DCNB, EPNP, BSP, 4-nitrobenzyl chloride, and 4-nitrobenzyl acetate was a substrate for this enzyme. Ethacrynic acid was a better substrate than l-menaphthyl sulfate. Cumene hydroperoxide was reduced at a considerable rate by this enzyme.
Abl (against GST Yrs-Yrs), Ab2 (against GST Ya-Yc), Ab3 (against GST Ybl-Yb2), and Ab4 (against GST Yp-Yp) contained 6 μg each of respective enzyme proteins purified from rat liver cytosol. Well 1 contained 6 μg of GST Yrs-Yrs. Wells GST Ybl-Yb2), and Ab4 (against GST Yp-Yp) contained sufficient amounts of IgG fractions of rabbit antisera. C, well 5 contained a used for B. Abl-Ab4 contained the same amounts of the anti-IgG fractions as with concomitant formation of GSSG from GSH.

**Immunochromic Properties of GST Yrs-Yrs**—An anti-IgG preparation from rabbit antisera raised against purified GST Yrs-Yrs caused significant precipitation against the antigen, but showed no cross-reactivity with GSTs Ya-Yc, Ybl-Yb2, and Yp-Yp, isolated as homogenous proteins from the rat liver cytosols, when examined by the Ouchterlony double-immunodiffusion test (Fig. 8A). Moreover, anti-IgG preparations from rabbit antisera raised against purified GSTs Ya-Yc, Ybl-Yb2, and Yp-Yp also showed no cross-reactivity with the enzyme GST Yrs-Yrs, whereas under the same conditions, the anti-GST Yrs-Yrs-IgG formed a significant precipitate against the antigen (Fig. 8B). These antibodies as well as that against GST Yrs-Yrs had all sufficient immunoreactivities with the corresponding purified GSTs as antigens (Fig. 8C).

**Tissue Distribution of GST Yrs-Yrs in the Rat**—Immunoblot analysis of various tissue cytosols of the male rats, carried out by using SDS-polyacrylamide gel electrophoresis and the anti-GST Yrs-Yrs-IgG preparation, suggested that testis contained a high concentration of GST Yrs-Yrs, comparable with that in the liver and also that the enzyme existed at somewhat lower concentrations in adrenal and kidney and at a much lower concentration in lung (Fig. 9). GST Yrs-Yrs, however, existed at extremely low concentrations in all the cytosols of the skin, heart, small intestine, and spleen, so that it could not be detected unless much larger amounts of these cytosolic proteins (at least more than five times of the liver protein) were applied.

**DISCUSSION**

So far as concerned with rat liver cytosolic GSTs, little is known of such an isozyme, except GST E (5-5), that is neither retained on the S-hexyl-SG affinity column (40), nor detected by the most widely used standard substrate, CDNB, or/and DCNB (30, 41). Extensive studies on the isozymes of GST in the rat liver have been made by using the affinity column and these standard substrates (41), and six major dimeric isozymes, GSTs Ya-Ya, Ya-Yc, Yc-Yc, Ybl-Ybl, Ybl-Yb2, and Yp-Yp, have been isolated and purified to homogeneity (28, 42). An additional homo-dimeric isozyme, GST P (GST Yp-Yp), was also isolated as a major GST, retainable on the affinity column and detected by CDNB, from the liver cytosol of rats bearing hepatic hyperplastic nodules induced by hepatocarcinogens (29, 43, 44). These seven rat liver GSTs are well studied on their primary structures (45), immunochromic properties (46), substrate specificities, tissue distribution, and species difference (41). Classification of these GSTs has been made in relation to human GSTs α-ω, μ, and ξ mainly based on their immunochromic homology and N-terminal amino acid sequences (46). The rat liver GSTs with subunit proteins Ya and Yc belong to class alpha, GSTs with Ybl and Yb2 to class mu, and GST P to class pi (46) (Table VI).

N-terminal amino acid sequences of GST subunit proteins in each class have a strong homology and can be readily differentiated from those of the other classes of GSTs. As to GST Yrs-Yrs, no homology was found in its N-terminal amino acid sequence and immunochromic property to any of the known rat GSTs. Furthermore, the Yrs subunit protein contained
protein Yn as a component of very minor heterodimeric the class alpha enzyme subunits Ya and Yc, but did not with those of the other classes of enzyme subunits (60). Similarly, the extrahepatic class mu enzymes reacted only with the Yb2 (46, 60).

found to be completely different from any of these three classes of GSTs in all respects so far as estimated from their chromatographic behaviors, immunochemical properties, substrate specificities, and N-terminal amino acid sequences (Table VI).

higher molar compositions of leucine and histidine residues than that of any other GST (Table II in the Miniprint).

GST Yα, isolated as a minor GST protein from the rat liver cytosol has been demonstrated by an immunochimuc study not to belong to class alpha nor to class mu, although no further attempt has been made to classify the isozyme by using the class pi enzyme GST P or an antibody against GST 5*-5*. GST Yα differs from GST Yα-Yrs in that it can be retained on the S-hexyl-SG affinity column and detected by CDNB with a low activity (much higher than for GST Yα-Yrs) and has high activities toward EPNP and 4-nitrophenyl acetate. The N-terminal amino acid sequence has not been shown with this GST isozyme.

Several different isozymes have been also found as major GSTs in the extrahepatic tissues of the rat, all of which had high catalytic activities toward CDNB similarly to the afore-mentioned isozyme in the normal rat kidney and small intestine (60, 66, 67). The class pi enzyme, GST P, exists rather as a major GSTs associating with the subunit proteins Ybl and Yb2 (51, 67). The subunit protein Yk reacted with the antibodies to GST E protein. GST Yrs-Yrs was also completely different in substrate specificity and in subunit M, and pl values from GST E (5-5), although both of them appeared in the flow-through fraction of the S-hexyl-SG affinity column chromatogram of the rat liver cytosol without being retained. GST E is the only reported GST isozyme that can neither be retained on the affinity column (68), nor show any catalytic activity toward CDNB and DCNB (30, 68-70). GST E has been demonstrated to be highly active toward the epoxide EPNP (30, 68-70), toward which GST Yrs-Yrs, however, had no activity, and to be completely inactive toward menaphthyl sulfate (69), the best substrate among examined sulfate esters for GST Yrs-Yrs (Table III). GST E has a subunit Mr value of 24,700 and pl value of 7.3 with the preparation from Sprague-Dawley rat liver cytosol (70) whereas the Mr and pl values of the hepatic subunit protein Yrs-Yrs from the Sprague-Dawley rat liver were 26,000 and 7.9, respectively. Little systematic study has been made on the immunochemical property of GST E, although antibodies raised against a few rat liver GSTs were demonstrated to show no cross-reactivity with this enzyme (30, 71). In addition, no information has been available on the N-terminal amino acid sequence of the GST E protein.

A GST isozyme, designated as 5*-5* because of its similarity to GST 5-5, has very recently been isolated from the nuclei of rat liver cells (72). This enzyme can neither be retained on the S-hexyl-SG affinity column nor cross-reactive with the antibodies against GSTs 1-2 and 3-4. GST 5*-5* has high activities toward EPNP and DNA hydroperoxide, but shows no appreciable activity toward CDNB. The N-terminal amino acid sequence has not been shown with this GST isozyme.

Earlier than 20 years ago, an attempt was made by Gillham (73) to prove the existence of an enzyme catalyzing GSH conjugation of benzyI and menaphthyl sulfates as putative precursors of the benzyI- and menaphthyl-mercapturic acids excreted into the urine of rats given the corresponding arylmethanols (74, 75). He found two GST isozymes active toward menaphthyl sulfate exist in the rat liver cytosol by an isoelectric focusing method and partially purified one of them to a purification fold of 76 (76). However, the partially purified
GSTs play a key role in preventing the formation of reactive metabolites of xenobiotics from their attacks on cellular biomolecules (41, 77, 78), which may induce necrosis and tumorigenesis of tissues. Either excessive formation or relatively low GST-mediated scavenging of the reactive metabolites may result in their covalent binding to DNA, leading to mutation or death of cells. A typical example is N-hydroxyacetylaminofluorene, a hepatocarcinogen, which has been demonstrated to be activated by phenol sulfotransferase IV (79, 80) and to be merely scavenged in nonenzymatic manner by GSH (81, 82). However, the active metabolites, sulfate esters of nonhepatocarcinogenic arylmethanols, are rapidly and completely scavenged by GSTs in the rat liver cytosol fortified with GSH (15–17, 22, 23), so that the carcinogens can neither bind covalently to DNA (16, 23) nor induce mutation of cells (15, 17, 22, 23) so far as examined in vitro.

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GSTs play a key role in scavenging the reactive sulfates of carcinogenic arylmethanols in the nontarget organ, rat liver, which has the highest level of hydroxysteroid sulfotransferases activating the carcinogen among all the examined tissues of the rat (24). Rat skin, a well-known target organ for 5-HCR (5), which has a sulfotransferase activity to activate the carcinogen, was found by the immunoblotting method to lack in GST Yrs-Yrs. Isolation and purification of the other rat liver GST isozymes active toward 5-HCR sulfates are in progress in our laboratory.

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REFERENCES

1. Cavalieri, E., Roth, R., Rogan, E., Grandjean, C., and Althoff, J. (1978) in Carcinogenesis (Jones, P. W., and Freudenthal, R. J., eds) Vol. 3, pp. 273–284, Raven Press, New York
2. Cavalieri, E., Roth, R., and Rogan, E. (1979) in Polynuclear Aromatic Hydrocarbons (Jones, P. W., and Leber, P., eds), pp. 91-92, Ann Arbor Science Publishers, Michigan
3. Seki, Y., and Fujita, H. (1980) in Carcinogenesis (Siga, S., T., ed) Vol. 5, pp. 1–31, Raven Press, New York
4. Hecht, S. S., Loy, M., and Hoffmann, D. (1976) in Carcinogenesis (Freudenthal, R. L., and Jones, P. W., eds) Vol. 1, pp. 255–260, Raven Press, New York
5. Amin, S., Juchat, A., Furuya, K., and Hecht, S. S. (1981) Carcinogenesis 2, 1027–1029
6. DiGiovanni, J., and Juchau, M. R. (1980) Drug Metab. Rev. 11, 61–101
7. Hecht, S. S., LaVoie, E. J., Marazita, R., Amin, S., Redenko, V., and Hoffmann, D. (1978) Cancer Res. 38, 2101–2104
8. DiPaola, A. (1976) in Chemical Carcinogens (Searle, C. E., ed) ACS Monograph 173, pp. 245–314, American Chemical Society, Washington, D.C.
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48. Tu, C.-P. D., and Reddy, C. C. (1985) J. Biol. Chem. 260, 9961-9964
49. Pickett, C., Telakowski-Hopkins, C. A., Ding, G.-J.-F., Argenbright, L., and Lu, A. Y. H. (1984) J. Biol. Chem. 259, 5182-5188
50. Telakowski-Hopkins, C. A., Rodkey, J. A., Bennett, C. D., Lu, A. Y. H., and Pickett, C. B. (1985) J. Biol. Chem. 260, 5820-5825
51. Álin, P., Jensen, C., Oeder, H., Farrell, H., and Mannervik, B. (1989) Biochem. J. 261, 531-539
52. Ding, G. J.-F., Lu, A. Y. H., and Pickett, C. B. (1985) J. Biol. Chem. 260, 13268-13271
53. Ding, G. J.-F., Ding, V.-D.-H., Rodkey, J. A., Bennett, C. D., Lu, A. Y. H., and Pickett, C. B. (1986) J. Biol. Chem. 261, 7952-7957
54. Abramovitz, M., and Listowsky, I. (1987) J. Biol. Chem. 262, 7770-7777
55. Ishikawa, T., Tsuchida, S., Satoh, K., and Sato, K. (1988) Eur. J. Biochem. 176, 551-557
56. Kispert, A., Meyer, D. J., Lalor, E., Coles, B., and Ketterer, B. (1988) Biochem. J. 260, 789-796
57. Lai, H.-C. J., Qian, B., Grove, G., and Tu, C.-P. D. (1988) J. Biol. Chem. 263, 11389-11395
58. Suguoka, Y., Kano, T., Okuda, A., Sakai, K., Kitagawa, T., and Muramatsu, M. (1985) Nucleic Acids Res. 13, 6049-6057
59. Jakoby, W. B., Ketterer, B., and Mannervik, B. (1984) Biochem. Pharmacol. 33, 2539-2540
60. Hayes, J. D., and Mantle, T. J. (1986) Biochem. J. 233, 779-788
61. Meyer, D. J., Lalor, E., Coles, B., Kispert, A., Álin, P., Mannervik, B., and Ketterer, B. (1989) Biochem. J. 260, 780-788
62. Tsuchida, S., Inumi, T., Shimizu, T., Ishikawa, T., Hatayama, I., Satoh, K., and Sato, K. (1987) Eur. J. Biochem. 170, 159-164
63. Hayes, J. D. (1986) Biochem. J. 233, 789-789
64. Hayes, J. D., and Chalmers, J. (1983) Biochem. J. 215, 581-588
65. Hayes, J. D. (1984) Biochem. J. 224, 839-852
66. Guthenberg, C., Jensen, H., Novotný, L., Österlund, E., Tahir, M. K., and Mannervik, B. (1985) Biochem. J. 230, 609-615
67. Tahir, M. K., Özer, N., and Mannervik, B. (1988) Biochem. J. 253, 759-764
68. Meyer, D. J., Christodoulides, L. G., Tan, K. H., and Ketterer, B. (1984) FEBS Lett. 237-230
69. Pickett, C. B., Hahne, H., and Jakoby, W. D. (1979) Biochem. Biophys. Res. Commun. 52, 1123-1128
70. Fjellstedt, T. A., Allen, R. H., Duncan, B. K., and Jakoby, W. B. (1973) J. Biol. Chem. 248, 3702-3707
71. Jakoby, W. B. (1978) Adv. Enzymol. 46, 383-414
72. Tan, K. H., Meyer, D. J., Gillies, N., and Ketterer, B. (1988) Biochem. J. 261, 841-840
73. Gillham, B. (1971) Biochem. J. 121, 667-672
74. Hyde, C. W., and Young, L. (1968) Biochem. J. 107, 519-522
75. Gillham, B., Clapp, J. J., Morrison, A. R., and Young, L. (1970) Biochem. J. 118, 24p
76. Gillham, B. (1973) Biochem. J. 135, 797-804
77. Chasseaud, L. F. (1976) in Glutathione: Metabolism and Function (Arias, I. M., and Jakoby, W. B., eds) pp. 77-114, Raven Press, New York
78. Jerina, D. M., and Bend, J. R. (1977) in Biological Reactive Intermediates (Jollow, D. J., Koscius, J. J., Snyder, R., and Vainio, H., eds) pp. 207-236, Plenum Press, New York
79. Wu, C.-C. G., and Straub, K. D. (1976) J. Biol. Chem. 251, 6529-6536
80. Sekura, R. D., and Jakoby, W. D. (1991) Arch. Biochem. Biophys. 211, 352-359
81. Ketterer, B., Coles, E., and Meyer, D. J. (1983) Environ. Health Perspect. 49, 59-69
82. Meyer, D. J., Beale, D., Tan, K. H., Coles, B., and Ketterer, B. (1985) FEBS Lett. 184, 139-143

SUGGESTED MATERIALS TO
A New Class of But Glutathione S-Transferase Yrs-Yrs Inactivating Reactive Sulfate Enters as Metabolite of Carcinogen-Antitumor Analogs

by

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Preparation of cytochrome fraction: Preparation of the tissue cytochrome fraction was carried out at 0-4°C as follows: Make Sepharose-4B (50% relative to the weight of the liver) and then kill by decapitation, separate portal vein and intrahepatic veins, and then homogenize in cold isotonic KCl to remove blood, and then control with scissors. The pooled skin preparation was then homogenized in isotonic KCl with a Polytron homogenizer (Microson, Switzerland). In addition, the size of the homogenate was well preserved in an emulsion of the homogenate (i.e., a mixture of 50% homogenate, 50% homogenate). Cytochrome fractions of the tissues were obtained by centrifugation. Protein concentrations of the cytochrome fractions were determined by the method of Lowry. The protein concentrations of the cytochrome fractions were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Preparative purification: GDP, NAD+, NADP, and NaF were incubated with a homogenate from the left liver lobe of the mouse normal 50% rate by the method of Niswanth and Jannasch (1968). NAD P (100 μM) was obtained from the liver lysate of male 380 rats bearing hepatic hyperplastic nodules by the methods of Salt (1962).

Hemoglobin: Glutathione S-transferases were rapidly isolated with 0.5M KCl and then purified by chromatography on the elution of the same lobe of the mouse normal 50% rate by the method of Niswanth and Jannasch (1968). GDP, NAD+, NADP, and NaF were incubated with a homogenate from the left liver lobe of the mouse normal 50% rate by the method of Niswanth and Jannasch (1968). NAD P (100 μM) was obtained from the liver lysate of male 380 rats bearing hepatic hyperplastic nodules by the methods of Salt (1962). Purification of GST by (Yrs-Yrs) from rat liver cytosol: All purification steps were done at 4°C. After the homogenate was obtained, the GST preparation, NAD P (100 μM), and glucose 6-phosphate buffer were added to the digestion buffer for 18 h against two volumes of the indicated buffer. Buffer A: 10 mM Tris-HCl (pH 8.5) containing 1 mM EDTA. Buffer B: 20 mM Tris-HCl (pH 8.5) containing 1 mM EDTA.
Glutathione S-transferase Yrs-Yrs

Amino acid analysis was performed in duplicate in the JEOl model JLC-300 amino acid analyzer after hydrolysis of 100 μg of GST Yrs-Yrs in 3N mercaptoethanesulfonic acid at 110°C for 18 h. Data are arithmetic mean values of two experiments. The amino acid composition was based on the assumption of the molecular weight of subunit Yrs as 26,000.

| Amino acid | (mol of amino acid/mol of protein) |
|------------|-----------------------------------|
| Asp | 22g |
| Thr | 6 |
| Ser | 17 |
| Gin | 35g |
| Pro | 9 |
| Gly | 23 |
| Ala | 25 |
| Cys | 1b |
| Val | 9 |
| Met | 5 |
| Ile | 3 |
| Leu | 33 |
| Tyr | 4 |
| Phe | 11 |
| His | 12 |
| Lys | 11 |
| Trp | 1 |

a) Value reflects the sum of aspartic acid plus asparagine (or glutamic acid plus glutamine) because the amide was quantitatively converted to the corresponding acid during the hydrolysis. 
b) Determined as cysteic acid after performic acid oxidation.

table II. The amino acid composition of GST Yrs-Yrs

**Fig. 1.** Fraction pattern of protein and GST activities from DE-52 column. A protein extract of 100,000 x g supernatant fraction (10 mg of protein) was applied to a DE-52 column (5 x 100 cm) equilibrated with buffer A. The column was washed with the same buffer and then eluted at a flow rate of 2.5 ml/min with a linear gradient of 0 to 300 mM NaCl in buffer A.

**Double-immunoassay.** Ouchterlony immunodiffusion was performed in 1% agar containing 0.1 M Tris-HCl buffer (pH 8.3), 0.1% NaCl and 0.9% (w/v) NaCl by the method of Ouchterlony (31).
