BIOCHEMICAL GENETIC STUDIES OF
THE GLUTATHIONE S-TRANSFERASES IN MAN

A thesis submitted for
the degree of Doctor of Philosophy of
the Australian National University

by

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STATEMENT

The studies presented in this thesis were completed by the author at the Department of Human Biology, John Curtin School of Medical Research, Australian National University. The assistance received from other persons is indicated in the list of acknowledgements. This work is otherwise original.

T. SUZUKI
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Finally, I wish to thank my parents, Katsumi and Aiko, and my brother, Yoshiaki for their unlimited encouragement.
ABSTRACT

1) The aims of the investigations presented in this thesis were to characterize the human glutathione S-transferase isozymes expressed in different tissues and to determine their genetic relationships.

2) The range in expression of the previously proposed glutathione S-transferase genes was investigated in various tissues by starch gel electrophoresis. The GST-1, GST-2 and GST-3 isozymes were found to be expressed in other human tissues in addition to liver and erythrocytes where these isozymes were originally reported by Board (1981) [Am. J. Hum. Genet. 33, 36-43]. Three additional forms of glutathione S-transferase were identified in several tissues. GST-4 was detected in skeletal and cardiac muscles and GST-5 in brain. GST-6 was detected in every tissue examined except erythrocytes.

3) The two apparently allelic products of the GST-1 locus, GST-1*1 and GST-1*2 have been purified from human livers. Both isozymes were found to have similar characteristics. Their subunit molecular weight, substrate affinity, heat stability, pH optimum and susceptibility to inhibition did not differ significantly. The present experiments further support the proposal that both GST-1*1 and GST-1*2 are the allelic products of the GST-1 locus in man.
4) The GST-2*1 and GST-2*2 isozymes were purified from human liver. Comparison of the subunit molecular weights, substrate affinities, kinetic studies, glutathione peroxidase activities, inhibition profiles and immunological properties showed that the GST-2*1 and GST-2*2 isozymes are very similar. However both GST-2*1 and GST-2*2 differ significantly from all the other isozymes characterized in these studies.

5) The GST-3 isozyme was purified from human lung. The subunit molecular weight, kinetic studies, inhibition studies and immunological comparisons showed that GST-3 is different from all the other isozymes purified in these studies and confirm that it is the product of an independent gene.

6) A muscle specific glutathione S-transferase isozyme, GST-4 was purified from human skeletal muscle. Although many biochemical properties of GST-4 were similar to those of GST-3, the subunit molecular weight of GST-4 was clearly different from that of GST-3 and the other GST isozymes characterized. Immunological comparisons with GST-1, GST-2 and GST-3 isozymes suggest that GST-4 is the product of an independent gene.

7) A brain specific glutathione S-transferase isozyme, GST-5 was purified from human brain. The GST-5 isozyme is
only expressed in brain, however it has an identical subunit molecular weight to that of GST-1 and also many of the biochemical characteristics of the GST-5 isozyme are similar to those of the GST-1 isozymes. In addition GST-5 and GST-1 show immunological identity. These data suggest that GST-5 is a product of the GST-1 gene which undergoes brain specific post-translational modification.

8) GST-6 was purified from human brain and characterized. GST-6 was found to be composed of subunits with different molecular weights. Biochemical and immunological studies suggest that GST-6 is not a modified product of either GST-3 or the other glutathione S-transferase genes reported in the present study, but the product of independent genes.
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</tr>
<tr>
<td>Bis</td>
<td>N,N'-Methylene-bis-acrylamide</td>
</tr>
<tr>
<td>BSP</td>
<td>Bromosulfophthalein</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-Chloro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>DCNB</td>
<td>1,2-Dichloro-4-nitrobenzene</td>
</tr>
<tr>
<td>EA</td>
<td>Ethacrynic acid</td>
</tr>
<tr>
<td>ENPP</td>
<td>1,2-Epoxy-3-(p-nitrophenoxy)propane</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione; ( \gamma )-glutamyl-cystenyl-glycine</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>( I_{50} )</td>
<td>Concentration of inhibitors producing 50% inhibition of enzyme activity</td>
</tr>
<tr>
<td>( K_m )</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>SDS</td>
<td>Lauryl sulfate, sodium salt</td>
</tr>
<tr>
<td>SRS-A</td>
<td>Slow reacting substance of anaphylaxis</td>
</tr>
<tr>
<td>tPB</td>
<td>trans-4-Phenyl-3-buten-2-one</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
</tbody>
</table>
Chapter 1

INTRODUCTION AND LITERATURE REVIEW
The glutathione S-transferases (EC 2.5.1.18) are a family of multifunctional proteins which are thought to execute at least three different roles in living organisms. First, the glutathione S-transferases catalyse the conjugation reaction between reduced glutathione (GSH; γ-glutamyl-cysteinyl-glycine) and various electrophiles, including many environmentally derived carcinogens and mutagens (Boyland and Chasseaud, 1969; Jakoby and Habig, 1980). These conjugates and/or their metabolites are excreted from the cell and are ultimately eliminated from the body in bile or urine. Secondly, the glutathione S-transferases are binding proteins (they have been termed 'ligandin') which are known to bind heme, bilirubin, bile acid, and steroid hormones. This function may influence the transport and intracellular solubility of these materials (Jakoby, 1978). Thirdly, a glutathione S-transferase may also catalyse the conjugation reaction between GSH and leukotriene A₄, an epoxide derived from arachidonic acid, to form leukotriene C₄ (a component of slow reacting substance A) (Samuelsson, 1983; Mannervik et al., 1984).
HISTORICAL BACKGROUND

Booth et al. (1961) and Combes and Stakelum (1961) were the first to simultaneously, but independently detect the presence of an enzyme which produces the conjugates of GSH and two different substrates, 3,4-dichloro-nitrobenzene and sulfobromophthalein. The enzyme has since been found to conjugate a number of substrates with GSH and has been designated as glutathione S-alkyltransferase, glutathione S-aryltransferase, glutathione S-aralkyltransferase, glutathione S-alkenetransferase, and glutathione S-epoxide transferase (Boyland and Chasseaud, 1969). Many of these terms are no longer used since the glutathione S-transferases in the species studied so far, have broad substrate specificity.

Three different hepatic binding proteins in rats, basic azo-dye carcinogen-binding protein, corticosteroid Binder I, and Y protein previously reported by several laboratories, were shown to be identical and the protein was called 'ligandin' by Litwack et al. (1971). Subsequently, Ketley et al. (1975) concluded that the protein described as 'ligandin' and glutathione S-transferase were identical in rats, following the extensive characterization of the catalytic activity and binding capacity of the glutathione S-transferases purified from rat liver (Fjellstedt et al., 1973; Habig et al., 1974a; Pabst et al., 1974). This finding therefore unified a wide body of information and indicated the multifunctional roles of the glutathione S-transferases.
Chapter 1. Introduction

THE CONJUGATION OF GSH

Generally, the metabolism of xenobiotics and drugs involves two phases, that is, 'phase I', biotransformation reactions including oxidations, reductions and hydrolyses, and 'phase II', conjugation reactions catalysed by a number of enzymes (Testa and Jenner, 1976; Caldwell, 1979).

Phase I reactions are carried out in the endoplasmic reticulum, or, in practical (experimental) procedures, in the microsomes which represent fragments of endoplasmic reticulum. Microsomes contain two electron transport chain systems which are known collectively as the microsomal monooxygenase system, that is, the NADPH-linked system with cytochrome P-450 as the terminal oxidase, and the NADH-linked system operating via cytochrome b₅. The cytochrome P-450 system is the main component of Phase I, and is responsible for the oxidation of numerous compounds. The microsomal cytochrome P-450 system has been extensively investigated and has been reviewed in detail (Coon and Persson, 1980; Wislocki et al., 1980).

Phase II reactions include glucuronidation by UDP-glucuronosyltransferase, methylation by methyltransferases, acetylation by N-acetyltransferase, sulfation by sulfotransferases, and glutathione conjugation by glutathione S-transferases. These reactions are carried out in both microsomes and/or the cytosol of cells.
Phase I reactions do not usually result in the loss of pharmacological activity or in increased water solubility but in some cases can result in products which are even more reactive than the original material (Caldwell, 1979). In contrast, the conjugates produced by phase II reactions are usually less reactive, more polar and thus can be readily excreted in bile or urine.

The conjugation of glutathione catalysed by the glutathione S-transferases is a component of the phase II reactions and is the first step in mercapturic acid formation (Figure I-1).

Numerous xenobiotics, representing a wide variety of electrophilic compounds including carcinogens and mutagens, are metabolised by this pathway and the resulting conjugates can be excreted from cells (Boyland and Chasseaud, 1969). The conjugates of GSH are substrates of γ-glutamyl transpeptidase, which converts them to S-substituted cysteinylglycine which is further cleaved to glycine and S-substituted cysteine by dipeptidase. The S-substituted cysteine is acetylated with acetylcoenzyme A to form N-acetyl S-substituted cysteine (mercapturic acid) by N-acetyltransferase (Meister 1983) (see Figure I-1).

The conjugates of glutathione in liver cells may be directly excreted in the bile which is stored in the gall bladder and is eliminated via the duodenum, or may be further converted by additional enzymatic reactions to mercapturic acids. The conjugates found in extrahepatic cells
Figure I-1. Schematic representation of the synthesis of mercapturic acids.

Abbreviations for Figure I-1:
AA, an amino acid; GST, glutathione S-transferase; γGTP, γ-glutamyl transpeptidase; RX, a hydrophobic compound having electrophilic group X.
The glutathione transferases are a group of intracellular binding proteins that play a crucial role in the metabolism and detoxification of endogenous and exogenous substances and in the disposal of toxic compounds into and out of cells, hence regulating the intracellular levels of glutathione (Lohse et al., 1970).

The diagram depicts a hypothesis where certain ligands are believed to be linked to the glutathione transferase, forming a complex which is then further processed by the enzyme dipeptidase to produce glutathione and glycine. This process is then followed by the reaction of N-acetyl cysteine-SR with glycine to produce glutamine and cysteine-SR. The resulting cysteine-SR is then used as a substrate by a specific enzyme to produce (N-acetyl)cysteine-SR.
can be transported via the blood stream and after further metabolism in the kidneys eliminated in urine (Meister, 1983).

**A BINDING PROTEIN**

The glutathione S-transferases may serve as intracellular binding proteins which bind to exogeneous and endogenous materials and may influence the passage of such compounds into and out of cells by regulating the intracellular levels of free and bound materials (Jakoby, 1978).

Arias et al. (1976) have proposed a hypothesis where rat ligandin (which is now known to be indentical to rat glutathione S-transferase B) is a major determinant of net organic anion flux from liver and kidney cells. Various organic anions examined include penicillin, bromosulfophalein, bilirubin, phenolsulfonphthlein, phlorizin, glutathione, p-amino hippurate, probenecid, conjugated bilirubin and bromosulfophthalein-glutathione conjugate (Levi et al., 1969; Kirsch et al., 1975). No similar studies have been carried out in man.

Ketley et al. (1975) found that rat glutathione S-transferase isozymes from liver had an affinity for hematin. Ketterer et al. (1976) reported that rat liver glutathione S-transferase was a heme-binding protein which possibly facilitated the transport of heme from mitochondria to the
cytoplasm in liver. This concept has been expanded by Harvey and Beutler (1982) who postulated that the glutathione S-transferase isozyme in erythrocytes functions physiologically as a hemin-binding and/or transport protein in developing erythroid cells.

Simons and Vander Jagt (1980) and Vander Jagt et al. (1983) reported the complex relationship between bilirubin binding and glutathione S-transferase activity and they found that the inhibition profiles of the enzyme activity by bilirubin were different for the glutathione S-transferase isozymes from human liver and placenta. These results seem to be attributable to differences in the subunit structure of these isozymes.

A KEY ENZYME IN LEUKOTRIENE SYNTHESIS

Arachidonic acid is a precursor of some potent mediators in the mammalian physiological system. The major metabolite groups of arachidonic acid are the prostaglandins and their derivatives, the thromboxanes in the cyclo-oxygenase pathway and the most recently discovered addition, the leukotrienes in the lipoxygenase pathway. The leukotrienes were discovered in leukocytes and their common structural feature is a conjugated triene (Samuelsson, 1983). The alternate pathways of arachidonic acid metabolism are shown in Figure I-2.
Figure I-2. Schematic representation of arachidonic acid metabolism.

Abbreviations for Figure I-2:
- GST, glutathione S-transferase; γGTP, γ-glutamyl transpeptidase; HHT, 12(S)-hydroxy-5,8,10-heptadecatrienoic acid; 5-HPETE, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; LTs A₄, B₄, C₄, D₄ and E₄; leukotrienes A₄, B₄, C₄, D₄ and E₄; PGs D₂, E₂, F₂α, G₂, H₂, and I₂; prostaglandins D₂, E₂, F₂α, G₂, H₂ and I₂; TXA₂, thromboxane A₂.
Prostaglandins act as vasodepressors in the cardiovascular system, and also affect smooth muscle tone. One prostaglandin may be involved in the mechanisms controlling the reproductive abilities of both males and females. Some prostaglandins and thromboxanes may be a significant factor in inflammatory processes (Giroud et al., 1977).

In the lipoxygenase pathway, the biosynthesis of the epoxide from arachidonic acid is carried out via the intermediate, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) by two enzymes, lipoxygenase and dehydrase, and the product is called leukotriene A₄. It is an unstable intermediate and a precursor of the physiologically active leukotrienes. Leukotriene A₄ is transformed into either leukotriene B₄ by a hydrolase, or leukotriene C₄ by a conjugation through C-6 to glutathione by an as yet undefined glutathione S-transferase. As shown in Figure I-2, leukotriene D₄ and E₄ are derived by the action of γ-glutamyl transpeptidase and dipeptidase on the peptide component of the conjugate.

Leukotrienes C₄, D₄ and E₄ have been identified as components of slow reacting substance of anaphylaxis (SRS-A), which seems to play a pathophysiological role in immediate hypersensitivity reactions. In addition they also have potent actions on gastro-intestinal, pulmonary and vascular smooth muscle but are not chemotactic. In comparison, leukotriene B₄ has potent chemotactic, chemokinetic and aggregatory actions on polymorphonuclear leukocytes of various species (Samuelsson, 1983).
Inhibitors of the lipoxygenase pathway have been examined because leukotrienes are involved in the pathogenesis of some conditions including asthma and hypersensitivity reactions (Salmon, 1983; Bach, 1984). Therefore, if the glutathione S-transferases are involved in leukotriene synthesis, then inhibitors of the glutathione S-transferases may be of clinical value.

**OTHER ENZYME ACTIVITIES**

Two additional enzyme activities of the glutathione S-transferases without GSH conjugate formation have been reported: i) $\Delta^5$-3-ketosteroid isomerase activity (steroid $\Delta$-isomerase) which catalyses the reaction from $\Delta^5$-3-ketosteroids to the corresponding conjugated $\Delta^4$-3-ketosteroids (Benson et al., 1977), and ii) glutathione peroxidase activity which catalyses the reduction of several organic hydroperoxides (Prohaska and Ganther, 1977). A reaction mechanism, like that of glutathione peroxidase (EC 1.11.1.9), has been proposed by Prohaska (1980). Glutathione S-transferase is thought to promote the nucleophilic attack by GSH on hydroperoxide resulting in a reactive intermediate, presumably the sulfenic acid of glutathione, GSOH:

$$\text{GSH} + \text{ROOH} \xrightarrow{\text{glutathione S-transferase}} \text{GSOH} + \text{ROH}$$

This sulfenic acid then reacts non-enzymatically with GSH
to produce oxidized glutathione (GSSG).

\[
\text{GSOH} + \text{GSH} \rightarrow \text{GSSG} + \text{H}_2\text{O} \quad \text{(Prohaska, 1980)}
\]

The biological significance of these additional enzyme activities of the glutathione S-transferases has not been clearly determined.

**DISTRIBUTION**

The glutathione S-transferases have been detected in animals, higher plants and certain bacteria (Jakoby and Habig, 1980). Glutathione S-transferase activity has been observed in almost all tissues investigated in vertebrates. In man and rats, the activity of the glutathione S-transferases has been shown to vary between tissues (Baar et al., 1981). The enzyme activity in liver and kidney is consistently higher than other tissues. This difference is presumably because these tissues appear to be major sites for the metabolism of xenobiotics.

Wahländer et al. (1979) examined the subcellular distribution of glutathione S-transferase between the mitochondrial matrix and cytosolic spaces in rat livers. These results indicated that 93% of the total enzyme activity was located in cytosolic spaces and 6.6% was in the mitochondrial matrix.
Microsomal glutathione S-transferase was purified from rat livers and a microsome-specific and several cytosolic isozymes have been detected in the microsomal fraction. The characteristics of the microsomal isozyme are different from cytosolic isozymes in rat livers, but, microsomal-specific isozymes have not yet been detected in man (Friedberg et al., 1979; Morgenstern et al., 1980 & 1982) Using immunochemical methods in rat liver, Morgenstern et al. (1983) found that the quantitative concentration of microsomal glutathione S-transferase was ten times higher than that of the cytosolic enzyme.

Immunohistochemical studies have been used to identify glutathione S-transferase in livers, kidneys and small intestines of rats, hamsters and man (Fleischner et al., 1977), and in the testes of mice (Lee, 1982) and rats (Redick et al., 1982; Ishii-Ohba et al., 1984) and also in human ovary (Tiltman, 1984).

Glutathione S-transferases purified from a variety of animals and plants are summarized in Table I-1.

THE DEVELOPMENTAL CHANGES IN GLUTATHIONE S-TRANSFERASE AND THE CONTROL OF THE ENZYME

The activity of the glutathione S-transferase in rat fetal liver and lung was found to be much less than that in the adult, and the activity of the enzyme rose until 30-40
Table I-1. List of various organisms (excluding man) from which glutathione S-transferase has been purified.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Organ</th>
<th>References</th>
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<tbody>
<tr>
<td>Earthworm (Lumbricidae)</td>
<td></td>
<td>Stenersen et al. (1979)</td>
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<tr>
<td>Moth 1 (Galleria mellonella)</td>
<td></td>
<td>Clark et al. (1977)</td>
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<td></td>
<td>(Wiseana cervinata)</td>
<td>Clark &amp; Drake (1984)</td>
</tr>
<tr>
<td>Cockroach (Periplaneta americana)</td>
<td></td>
<td>Clark et al. (1973)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Usui et al. (1977)</td>
</tr>
<tr>
<td>House fly (Musca domestic)</td>
<td></td>
<td>Clark et al. (1973)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Motoyama &amp; Dauterman (1977)</td>
</tr>
<tr>
<td>Fly (Drosophila melanogaster)</td>
<td></td>
<td>Jansen et al. (1984)</td>
</tr>
<tr>
<td>Grass grub (Costelytra zealandica)</td>
<td></td>
<td>Clark et al. (1973)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clark et al. (1984; 1985)</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>Liver</td>
<td>Ramage &amp; Nimmo (1983)</td>
</tr>
<tr>
<td>Chicken</td>
<td>Liver</td>
<td>Yeung &amp; Gidari (1980)</td>
</tr>
<tr>
<td>Shark</td>
<td>Liver</td>
<td>Sugiyama et al. (1981)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Liver</td>
<td>Lee et al. (1981)</td>
</tr>
<tr>
<td></td>
<td>Intestine</td>
<td>Parchment &amp; Benson (1984)</td>
</tr>
<tr>
<td>Rat</td>
<td>Liver</td>
<td>Pabst et al. (1973)</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>Dierickx (1983)</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>Ishikawa &amp; Sies (1984)</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>Tu et al. (1984)</td>
</tr>
<tr>
<td>Hamster</td>
<td>Liver</td>
<td>Smith et al. (1980)</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>Liver</td>
<td>Irwin et al. (1980)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Liver</td>
<td>Gawai &amp; Pawar (1984)</td>
</tr>
<tr>
<td>Pig</td>
<td>Liver</td>
<td>Grahnén &amp; Sjöholm (1977)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>Trip et al. (1974)</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>Asaoka &amp; Takahashi (1983a)</td>
</tr>
</tbody>
</table>

1: the enzyme was purified from larvae of moths
days of age when it reached its maximum (Klaassen, 1975; Mukhtar and Bresnick, 1976). In mice, Rouet et al. (1984) have reported that the developmental patterns of glutathione S-transferase activity were similar in brain, lung and liver during prenatal and postnatal life.

So far, very little data has been published relating to developmental changes of glutathione S-transferases in man. Mukhtar et al. (1981) reported the quantitative estimation of glutathione S-transferase activity in fetal and adult tissues. They found that glutathione S-transferase activity in certain fetal tissues was higher than that in corresponding adult tissues, however because of wide interindividual variations in the tissue enzyme activities, these differences were not significant. Warholm et al. (1981a) and Strange et al. (1984b) examined the variation
Chapter 1. Introduction

of the glutathione S-transferases from fetal liver using isoelectric focusing and starch gel electrophoresis respectively. They failed to find the GST-1 (or near-neutral) isozyme in liver during fetal life. In contrast they found that the GST-2 (or cationic) isozyme and the GST-3 (or anionic) isozyme were dominant in fetal liver although GST-3 activity was weak in adult liver (Warholm et al., 1981a; Strange et al., 1984b).

The glutathione S-transferases seem to be affected by endogeneous and exogeneous factors (Chasseaud, 1979). Like another drug induced enzyme, cytochrome P-450, phenobarbital and 3-methylcholanthrene were initially used to induce glutathione S-transferase (Klaassen and Plaa, 1968; Reyes et al., 1969; Klaassen, 1975; Hales and Neims, 1977). Other chemicals have also been shown to act as inducers of glutathione S-transferase in both liver and extrahepatic organs (Table I-2). At least one hormone, thyroid hormone, was found to be one of the hormonal regulators of the hepatic glutathione S-transferase (Y protein) in rats (Reyes et al., 1971). The hypothalamic-hypophyseal-gonadal regulation of hepatic glutathione S-transferases was examined by Lamartiniere (1981) who proposed that a hypothalamic inhibiting factor may cause sexual differentiation of the enzyme in rat liver.
Table I-2. Chemicals used as inducers of the glutathione S-transferases in rats and mice.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>References</th>
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<tbody>
<tr>
<td>Polychlorinated biphenyl mixture</td>
<td>(Marniemi et al., 1977)</td>
</tr>
<tr>
<td>DDT</td>
<td>(Chasseaud, 1979)</td>
</tr>
<tr>
<td>trans-Stilbene oxide and its metabolites</td>
<td>(Seidegard et al., 1979)</td>
</tr>
<tr>
<td>Monascus pigments</td>
<td>(Sako et al., 1983)</td>
</tr>
<tr>
<td>Diethylnitrosoamine + 2-Acetylaminofluorene + Partial heptatectomy</td>
<td>(Kitahara et al., 1983)</td>
</tr>
<tr>
<td>Propylthiouracil</td>
<td>(Kariya et al., 1984)</td>
</tr>
<tr>
<td>Cyclic 12-, 8- &amp; 6-carbon compounds</td>
<td>(Sparnis et al., 1984)</td>
</tr>
<tr>
<td>2(3)-tert-Butyl-4-hydroxyanisole</td>
<td>(Benson et al., 1978)</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>(Adams et al., 1985)</td>
</tr>
</tbody>
</table>

METHODS FOR DETECTING GLUTATHIONE S-TRANSFERASE ACTIVITY

Habig and Jakoby (1981) reviewed several spectrophotometric assay systems for the glutathione S-transferases which determine the rate of thioether and thioester formation. Asaoka and Takahashi (1983b) reported another new spectrophotometric method using o-dinitrobenzene as a substrate. In addition, Seidegard et al. (1984) described a radiochemical assay using $[^3H]$trans-stilbene oxide as a substrate. The most sensitive spectrophotometric method for every glutathione S-transferase isoynme studied so far is performed in the presence of reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB). The product, S-
2,4-dinitrophenyl glutathione can be readily detected by its absorbance at 340 nm.

Several methods designed to detect the glutathione S-transferases after gel electrophoresis have been described and they are listed in Table I-3. So far three methods using polyacrylamide gel electrophoresis with several staining procedures have been reported. However, the isozymes of glutathione S-transferase from crude tissue extracts cannot be clearly detected by these methods. New staining methods are required if any gel matrix other than starch is used for the electrophoretic separation of glutathione S-transferase from tissue extracts. The method for detecting glutathione S-transferase activity after electrophoresis in starch gels was invented independently by Board (1980) and Scott and Wright (1980). This method relies on the inhibition of the blue 'starch-iodine' reaction by free GSH. In zones of enzyme activity all GSH applied to the gel is conjugated to CDNB and these zones can be identified by the development of an intense blue stain when an iodine solution is applied to the gel surface. At the present stage, starch gel electrophoresis with the histochemical staining technique described by Board (1980) and Scott and Wright (1980) is the only useful technique to detect isozymes of the glutathione S-transferases after electrophoresis.
Table I-3. Methods to detect glutathione S-transferase on gel electrophoresis

<table>
<thead>
<tr>
<th>Electrophoresis</th>
<th>Staining method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>(GSH + CDNB) &amp; iodine-starch</td>
<td>Board (1980)\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Scott &amp; Wright (1980)\textsuperscript{b}</td>
</tr>
<tr>
<td>Polyacrylamide</td>
<td>GSH + CDNB</td>
<td>Kenney &amp; Boyer (1981)\textsuperscript{c}</td>
</tr>
<tr>
<td></td>
<td>(GSH + CDNB) &amp; methyl iodide</td>
<td>Clark (1982)\textsuperscript{d}</td>
</tr>
<tr>
<td></td>
<td>(GSH + CDNB) &amp; nitro-tetrazolium salt</td>
<td>Ricci et al. (1984)\textsuperscript{e}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}: Electrophoresis was carried out at pH 8.6 and 6.0.
\textsuperscript{b}: Electrophoresis was performed at pH 7.5.
\textsuperscript{c}: Isoelectric focusing was carried out. After staining, the gel was scanned for the appearance of product, S-(2,4-dinitrophenyl)glutathione.
\textsuperscript{d}: After polyacrylamide gel electrophoresis and isoelectrofocusing were performed, glutathione S-transferase activity with alkyl, aryl, or aralkyl iodide substrates gave rise to blue zones in the gels containing small amounts of starch after oxidation by hydrogen peroxide of the iodide released during the transferase reaction.
\textsuperscript{e}: The staining method is based on the fast reduction of nitroblue tetrazolium salt by residual glutathione.

GENETICS OF HUMAN GLUTATHIONE S-TRANSFERASE

The human glutathione S-transferases were first purified from liver by Kamisaka et al. (1975) and these basic isozymes were designated as glutathione S-transferases \( \alpha \), \( \beta \), \( \gamma \), \( \delta \) and \( \varepsilon \) according to their increasing isoelectric points. These isozymes had fairly similar characteristics such as an identical molecular weight (48,500), two identi-
Chapter 1. Introduction

cal subunits, similar catalytic activities with various substrates, similar amino acid composition and immunological cross-reactivity. Therefore Kamisaka et al. (1975) concluded that these isozymes were due to charge isomers of the enzyme arising from deamination \textit{in vivo}.

A single anionic form of glutathione S-transferase (glutathione S-transferase $\rho$) was purified from human erythrocytes by Marcus et al. (1978) and it was found to have several characteristics that differed from the isozymes in liver. Subsequently another anionic form of glutathione S-transferase (glutathione S-transferase $\pi$) was purified from placenta by Guthenberg et al. (1979). Because both anionic forms had very similar properties, Guthenberg and Mannervik (1981) concluded that the isozyme ' $\pi$ ' from placenta was identical with, or very closely related to, the isozyme ' $\rho$ ' from erythrocytes. Koskelo et al. (1981) purified an anionic form of glutathione S-transferase from human lung, and they observed a marked difference in the type of inhibition by bilirubin between it and glutathione S-transferase $\rho$ from erythrocytes. However, more recently, amino acid sequence analysis of the N-terminal region of the placental and lung isozymes was reported and the results revealed that the polypeptide sequence of the placental isozyme was identical with that of the lung enzyme (Dao et al., 1984). Two additional anionic forms of glutathione S-transferase ($\omega$ and $\psi$) were purified from human liver by Awasthi et al. (1980) and the catalytic
properties of glutathione S-transferase \( \omega \) were significantly different from those of the cationic forms from liver, although antibodies against the cationic forms cross-reacted with glutathione S-transferase \( \omega \).

Warholm et al. (1981a & 1983) reported the purification of a near-neutral form of the glutathione S-transferase (\( \mu \)) from liver and found that its molecular and catalytic properties were different from other previously purified isozymes.

The variations in the glutathione S-transferases previously reported by several research groups are summarized in Table I-3. The pl values and molecular weights of subunits are described according to the primarily reported results for each form.

As mentioned before, Board (1980) and Scott and Wright (1980) simultaneously reported a similar novel histochemical method to identify glutathione S-transferase isozymes after electrophoresis. Although both researchers confirmed the previous reports that human erythrocytes contain only one form of glutathione S-transferase, Board (1980 & 1981a) suggested that the most active forms of glutathione S-transferase in liver are the products of two autosomal loci, GST-1 and GST-2. Board (1981a) also suggested that these loci were polymorphic and interestingly, described a common null allele at the GST-1 locus. The acidic isozyme identified in erythrocytes was thought to be the product of an additional locus termed GST-3 (Board, 1981a).
Table I-4. The variation of the glutathione S-transferases from human tissues.

<table>
<thead>
<tr>
<th>Isozymes&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tissues</th>
<th>pI</th>
<th>Subunit M.W.&lt;sup&gt;b&lt;/sup&gt;</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>α, β, γ, δ, ε</td>
<td>liver</td>
<td>7.8-8.8</td>
<td>24,250</td>
<td>Kamisaka et al. (1975)</td>
</tr>
<tr>
<td>µ</td>
<td>liver</td>
<td>6.6</td>
<td>26,300</td>
<td>Warholm et al. (1983)</td>
</tr>
<tr>
<td>π</td>
<td>placenta</td>
<td>4.8</td>
<td>23,400</td>
<td>Guthenberg &amp; Mannervik (1981)</td>
</tr>
<tr>
<td>ρ</td>
<td>erythrocytes</td>
<td>4.5</td>
<td>23,750</td>
<td>Marcus et al. (1978)</td>
</tr>
<tr>
<td>ψ</td>
<td>liver</td>
<td>5.4</td>
<td>22,500</td>
<td>Awasthi et al. (1980)</td>
</tr>
<tr>
<td>ω</td>
<td>liver</td>
<td>4.6</td>
<td>22,500</td>
<td>Awasthi et al. (1980)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mannervik (1985) proposed three isozymes according to differences of isoelectric points and other properties.  
Basic isozyme: 8<pI<9  
Near-neutral isozyme: pI=6.6  
Acidic isozyme: pI<5.

<sup>b</sup> Each isozyme was reported to be a homo-dimer in man.

Several research groups have subsequently adopted Board's genetic model and reported the frequency of alleles at the different GST loci in a number of populations. These results are summarized in Table I-5 for alleles at the GST-1 locus and Table I-6 for alleles at the GST-2 locus. However, two research groups, Strange et al. (1984b) and Laisney et al. (1984), did not agree that the variations of the GST-2 locus were allelic products according to Board's proposal (1981a & b), and suggested that those variations are the product of post-translational changes.
Unfortunately, because GST-2 is not expressed in readily biopsied tissues, formal genetic studies cannot be undertaken to resolve this question.

Silberstein and Shows (1982) attempted to identify the chromosomal locations of the GST loci by the use of human-mouse somatic cell hybrids and the electrophoretic technique described by Board (1980). These authors assigned the GST-1 locus to chromosome 11 but could not distinguish the products of the other GST loci in the hybrid cell lines. Laisney et al. (1983) also studied the chromosomal locations of the GST loci using a technique similar to that of Silberstein and Shows (1982), and reported that the GST-3 locus was located on chromosome 11. Independently Suzuki and Board (1984) re-evaluated the electrophoretic analysis carried out by Silberstein and Shows and concluded that the isozyme mapped to chromosome 11 was not GST-1 but GST-3 (see Appendix I, pp.186-187). It now appears clear that the GST-3 locus is on chromosome 11 and the other GST loci have yet to be mapped.

**THE AIM OF THIS STUDY**

The glutathione S-transferases are currently of great interest in cancer research, physiology and toxicology. It has also been suggested that the glutathione S-transferases are of importance in a number of pathological conditions. Because of their experimental suitability, the basic concepts of the glutathione S-transferase system have been
Table I-5. The gene frequencies of the alleles at the GST-1 locus in various populations.

<table>
<thead>
<tr>
<th>RACE</th>
<th>GST-1*1</th>
<th>GST-1*2</th>
<th>GST-1*0</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese (n=96)</td>
<td>0.1709</td>
<td>0.0646</td>
<td>0.7645</td>
<td>Board (1981a)</td>
</tr>
<tr>
<td>Indian (n=43)</td>
<td>0.1614</td>
<td>0.2790</td>
<td>0.5596</td>
<td>Board (1981a)</td>
</tr>
<tr>
<td>Australian Caucasian (n=40)</td>
<td>0.1061</td>
<td>0.0784</td>
<td>0.8154</td>
<td>Board (1981a)</td>
</tr>
<tr>
<td>European Caucasian (n=29)</td>
<td>0.13</td>
<td>0.23</td>
<td>0.64</td>
<td>Strange et al. (1984b)</td>
</tr>
<tr>
<td>French Caucasian (n=32)</td>
<td>0.115</td>
<td>0.455</td>
<td>0.43</td>
<td>Laisney et al. (1984)</td>
</tr>
<tr>
<td>Japanese (n=118)</td>
<td>0.2411</td>
<td>0.0934</td>
<td>0.6655</td>
<td>Akiyama &amp; Abe (1984)</td>
</tr>
<tr>
<td>Singapore Chinese (n=221)</td>
<td>0.1558</td>
<td>0.1117</td>
<td>0.7274</td>
<td>Bhattacharyya &amp; Saha (1984)</td>
</tr>
</tbody>
</table>

a: a new allele, GST-1*3 was reported with a frequency of 0.0050.

Table I-6. The gene frequencies of the alleles at the GST-2 locus in various populations.

<table>
<thead>
<tr>
<th>RACE</th>
<th>GST-2*1</th>
<th>GST-2*2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese (n=96)</td>
<td>0.8125</td>
<td>0.1875</td>
<td>Board (1981a)</td>
</tr>
<tr>
<td>Indian (n=43)</td>
<td>0.7791</td>
<td>0.2209</td>
<td>Board (1981a)</td>
</tr>
<tr>
<td>Australian Caucasian (n=40)</td>
<td>0.8375</td>
<td>0.1625</td>
<td>Board (1981a)</td>
</tr>
<tr>
<td>French Caucasian (n=32)</td>
<td>0.648</td>
<td>0.348</td>
<td>Laisney et al. (1984)</td>
</tr>
<tr>
<td>Japanese (n=118)</td>
<td>0.7924</td>
<td>0.2076</td>
<td>Akiyama &amp; Abe (1984)</td>
</tr>
<tr>
<td>Singapore Chinese (n=221)</td>
<td>0.7671</td>
<td>0.2149</td>
<td>Bhattacharyya &amp; Saha (1984)</td>
</tr>
</tbody>
</table>

a: the variation was concluded to be the result of post-translational modification.
b: a null allele was reported and its gene frequency was 0.0181.
derived from experiments using rats. In comparison, at the time when this project was initiated much less was known about the biochemistry and genetic control of the human glutathione S-transferases. Since the nature of the glutathione S-transferases in man may differ significantly from those in rats, it is important that the biochemical genetic relationships of the human isozymes be further investigated.

As described earlier, several research groups have purified the isozymes of glutathione S-transferase from human tissues and have described their varying characteristics. However the origin of the observed variation and the relationships between different purified isozymes are not clear. In contrast, the proposals of Board (1981a & b) have provided a genetic model which, to a large degree, clarifies the relationships between the various isozymes that can be identified electrophoretically.

The aim of the present investigations has been to characterize the products of the various human glutathione S-transferase genes proposed by Board (1981a & b), in order to further evaluate the validity of Board's model and where possible to relate those isozymes to the forms previously characterised from various tissues by different authors. These biochemical genetic studies are essential to the understanding of the roles played by the glutathione S-transferases in the detoxification of carcinogens and mutagens and in other functions. Furthermore, these studies are important in understanding the genetic susceptibility of individuals to some diseases, for example, cancer and asthma, in man.
Chapter 2

Tissue heterogeneity of glutathione S-transferase expression in man

Several glutathione S-transferases with different isoelectric points have been identified in various human tissues, for example, in the liver of the 1970s (Sasaki et al., 1980) and erythrocytes (Kandas et al., 1979) and in other tissues (Carnita et al., 1979).

In this chapter, additional tissue samples have been investigated to determine the range in expression of the previously proposed glutathione S-transferase genes and to determine if any additional tissue-specific glutathione S-transferase isozymes are present in man.
INTRODUCTION

Several glutathione S-transferases with different isoelectric points have been identified in various human tissues, for example, in liver (Kamisaka et al., 1975; Awasthi et al., 1980), erythrocytes (Marcus et al., 1978), and placenta (Gutenberg et al., 1979).

Board (1981a) examined the glutathione S-transferase expression in human livers and erythrocytes by starch gel electrophoresis and suggested that the glutathione S-transferase isozymes are the product of at least three loci, GST-1, GST-2, and GST-3. Two loci, GST-1 and GST-2, were thought to be polymorphic and the data suggested that there is a common null allele at the GST-1 locus. The products of both the GST-1 and GST-2 loci were reported to be the major contributors to the total glutathione S-transferase activity in liver. In comparison, the major GST isozyme found in erythrocytes was thought to be the product of the GST-3 locus and is not electrophoretically polymorphic (Board, 1981a & b).

In this chapter additional human tissues have been investigated to determine the range in expression of the previously proposed glutathione S-transferase genes and to determine if any additional tissue specific glutathione S-transferase isozymes are present in man.
MATERIALS: Human tissues (liver, brain, lung, heart, muscle, kidney, spleen, colon and erythrocytes) were obtained from six adult males and five adult females at autopsy from Royal Canberra Hospital. All samples were stored below -20°C.

SAMPLE PREPARATION: Samples were homogenized in 50 mM Tris/HCl buffer, pH 7.4, and centrifuged at 1,500 x g for 10 minutes. The supernatants of the the homogenates absorbed on small pieces of filter paper (Whatman No.3MM or No.17, 0.5 cm x 0.5-0.7 cm) were inserted into starch gels along a predetermined line at suitable intervals.

STARCH GEL ELECTROPHORESIS: Horizontal gel electrophoresis was carried out by the method of Board (1980). An 11% starch gel was prepared in a gel buffer, which was a 1:10 dilution of the TEB buffer (0.9 M Tris/0.02 M ethylenediaminetetra-acetic acid (EDTA-acid form)/0.5 M boric acid, pH 8.6). The electrode buffer was a 1:7 dilution of the TEB buffer (pH 8.6). Electrophoresis was performed at approximately 5°C between cooling blocks for 8-12 hours at 5-10 V/cm. After electrophoresis the gel was sliced horizontally into two equal halves and usually the bottom half was used for staining.

STAINING: The activity of the glutathione S-transferases was detected by a two stage staining procedure.
Stage 1: the specific enzyme reaction utilizing 1-chloro-2, 4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates.

Stage 2: Utilizing the colour reaction of starch-iodine (blue colour) and its inhibition by GSH.

A filter paper sheet (Whatman No.3MM, 14 cm wide x 15 cm long) was saturated with the reaction mixture containing 8 mg of CDNB (which was dissolved in 0.8 ml ethanol at 37°C), 14 mg of GSH and 20 ml of 0.1 M sodium phosphate buffer (pH7.5) preincubated at 37°C. The sheet was overlayed on the surface of the gel and incubated at 37°C for 1-2 hours. The filter paper was then removed and the second stage staining solution containing 0.9 ml of 1%(w/v) I₂ in 2%(w/v) KI in 30 ml of distilled water and 30 ml of 2%(w/v) molten agar was poured gently over the surface. The position of the enzyme was visualized by the development of an intense blue starch-iodine reaction on the gel surface where GSH had been conjugated to CDNB.

RESULTS

The glutathione S-transferase isozymes were identified by their electrophoretic mobility in starch gels as shown in Figures II-1, 2 and 3. Unfortunately every tissue was not available from each subject studied. In addition every isozyme present in a particular tissue in one individual was not necessarily expressed in the same tissues from
Figure II-1. Electrophoretic patterns of glutathione S-transferase isozymes in livers at pH 8.6

A) The anodal side from the origin
Lane 1: GST-1*0 and GST-3 phenotypes
Lane 2: GST-1*1 and GST-3 phenotypes
Lane 3: GST-1*2 and GST-3 phenotypes
Lane 4: GST-1*2-1 and GST-3 phenotypes

B), C) and D) The cathodal side of the origin
Various GST-2 phenotypes including the isozymes termed GST-2*1 and GST-2*2
Figure II-2. Electrophoretic patterns of new isozymes in several tissues at pH 8.6.
Figure II-3.

Electrophoretic patterns of glutathione S-transferase isozymes in various tissues from two individuals.

A) Includes various tissues from case #135 and brain from case #134.

B) Includes various tissues from case #48/84.
   Note the different GST-2 phenotypes in kidney and liver.
Table II-1. Tissue distribution of the glutathione S-transferase isozymes.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>(Number tested)a</th>
<th>Number observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GST-1</td>
<td>GST-2</td>
</tr>
<tr>
<td>Liver (n=11)</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Brain (n=6)</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Lung (n=9)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Heart (n=11)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Spleen (n=7)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Kidney (n=8)</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Muscle (n=9)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Stomach (n=5)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Colon (n=6)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Erythrocytes (n=11)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a: Number studied in the present experiments. Different numbers of tissues were obtained from 11 individuals.
b: Trace amounts detected.

other cases. A summary of the isozymes observed in each tissue is shown in Table II-1.

GST-1 (relatively slow components migrating towards the anode at pH 8.6, Figure II-1A): The GST-1 isozymes were found in every tissue type studied except erythrocytes. However, in many individuals, the products of the GST-1 locus could not be detected in any tissue. In individuals where GST-1 was present, it was found to be most active in the liver. When GST-1 isozymes were not detected in an
individual liver sample, they were also absent from other tissues from the same individual.

GST-2 (usually triplet patterns migrating toward the cathode at pH8.6, Figures II-1B, C & D): GST-2 isozymes were found in every tissue type examined except erythrocytes. However, in many individuals, GST-2 was absent or very weak in many tissues. In addition the relative staining intensity of the different bands was found to vary between different tissues from the same individual. GST-2 was most active in the liver and kidney.

GST-3 (an electrophoretically fast component migrating toward the anode at pH8.6, Figures II-1A & II-3): The GST-3 isozyme was found in every tissue and every individual investigated. However, the level of expression of GST-3 varied considerably. GST-3 was found to be the most active form of glutathione S-transferase in the lung and the least active in the liver.

Several tissues contained glutathione S-transferase isozymes which could not be clearly identified as the products of the three previously described loci, GST-1, GST-2 and GST-3 (Figures II-2 & II-3).

Both skeletal and cardiac muscle samples expressed a glutathione S-transferase isozyme which migrated toward the anode slightly in advance of haemoglobin A and was not found in tissues other than muscle. The isozyme was present in all individuals including those who were homozygous for the null allele (GST-1*0) at the GST-1 locus. Figure II-2
shows the relative mobility of muscle specific glutathione S-transferase in comparison with the products of the GST-1 and GST-3 loci. This new isozyme has been termed GST-4.

An additional glutathione S-transferase isozyme, which migrates towards the anode between the two allelic products of GST-1 locus (GST-1*1 and GST-1*2) has been identified in brain tissue. This isozyme is shown in Figure II-2 and has been termed GST-5.

An electrophoretically fast form of glutathione S-transferase which migrates towards the anode in front of GST-3 has been detected in all the tissue types studied except erythrocytes. This isozyme was not always observed and was only rarely detectable in the liver. This isozyme is shown in Figure II-2 in brain tissue, skeletal muscle and cardiac muscle from a single individual and has been termed GST-6.

DISCUSSION

GST-1, GST-2 and GST-3 isozymes were found to be expressed in other human tissues in addition to liver and erythrocytes in which these isozymes have previously been reported by Board (1981a & b). These data also largely confirm the findings of two independent reports published recently by Strange et al. (1984b) and Laisney et al. (1984).

Many individuals did not express GST-1 isozymes, in any tissues studied, presumably because of the homozygous
inheritance of a null allele at the GST-1 locus proposed by Board (1981a). Because of this apparently common deficiency, it was not clear if the absence of GST-1 isozymes from a tissue was due to the fact that GST-1 was not a normal product of that tissue or if it was a case of genetic deficiency. However one individual expressed GST-1 in all the tissues studied except erythrocytes. This confirmed that if GST-1 was expressed in the liver, it could also be expressed in other tissues. Furthermore, it is clear from the data presented in Table II-1 that even if GST-1 isozymes were expressed in the liver, they were not always expressed in other tissues from the same individual. This observation suggests that the expression of GST-1 in some tissues may not only depend on the inheritance of the GST-1*1 or GST-1*2 alleles but also on specific induction. Strange et al. (1984b) and Laisney et al. (1984) reported that several other tissues including platelets, cultured fibroblasts and lymphoblastoid cells do not express the products of the GST-1 locus. Furthermore the products of the GST-1 locus were not observed in fetal livers which included 6 samples of 10-13 weeks, 12 samples of 14-26 weeks and 10 samples of 26-40 weeks (Strange et al., 1984b).

The two apparently allelic products of the GST-1 locus were also found in the present experiments. As described in Chapter 1, several additional research groups have also confirmed the proposal of Board (1981a & b) that the GST-1 locus is polymorphic with a common null allele.
In the present study, the products of the GST-2 locus were also detected in every tissue studied except erythrocytes. Strange et al. (1984b) reported that platelets, cultured fibroblasts and lymphoblastoid cells did not contain the GST-2 isozymes. In comparison, Laisney et al. (1984) failed to find expression of GST-2 isozymes in muscle, heart, skin and brain. Although the level of activity was in some cases quite low, GST-2 isozymes were clearly observed in skeletal muscle, cardiac muscle and brain in the present study.

Board (1981a) reported that the cationic glutathione S-transferase isozymes in human liver were the products of a locus termed GST-2. It was also suggested that this locus was polymorphic with two alleles. In contrast, Strange et al. (1984b) and Laisney et al. (1984) showed conflicting results, where, unlike the results of Board (1981a), the observed phenotype frequencies were not in agreement with a Hardy-Weinberg equilibrium. They concluded that the variation in cationic isozymes were due to post-synthetic modification of the product of the GST-2 locus rather than allelic variation. More recently, Stockman et al. (1985) purified two cationic isozymes which seem to be identical to the allelic products of the GST-2 locus originally proposed by Board (1981a & b). However, they found that the two cationic isozymes were immunologically different. Therefore the results of Stockman et al. (1985) suggest that the different cationic isozymes are the
products of different genes rather than allelic variation or post-translational modification of a single gene product.

Strange *et al.* (1984a) reported one adult liver specimen, with GST-1 and GST-3 activity, but a complete absence of GST-2 activity. Since GST-1 and GST-3 activities were present, it is unlikely that glutathione S-transferase activity has been inactivated and this individual may represent a case of GST-2 deficiency. This case may be of importance in determining the number of genes or alleles that control the cationic GST-2 isoenzymes. Since two different GST-2 isoenzymes have been purified and characterized in later experiments, further discussion of these aspects is contained in Chapter 4.

The product of GST-3 was observed in every tissue although different levels of expression were present. GST-3 appears as a single band on starch gel electrophoresis using the buffer at pH8.6 described in the present experiments. However, Strange *et al.* (1983) demonstrated two forms of glutathione S-transferase in erythrocytes when starch gel electrophoresis was performed with Tris/Citrate buffer (pH7.5). They concluded that the two enzyme forms in erythrocytes result from post-synthetic modification. On the other hand, Laisney *et al.* (1984) concluded that the isozyme in erythrocytes (which they called GST'e') was different from the product of the GST-3 locus which was observed in all other tissues when the electrophoresis was
carried out at pH 6.0 or 8.0 using the method of Board (1980). The basis for Laisney et al.'s suggestion that erythrocyte glutathione S-transferase is different from GST-3 in other tissues resulted from their observation that the electrophoretic mobility of these two isozymes differed slightly. Board (1981a) also noted this difference and further studies are therefore needed to determine if the GST isozyme in erythrocytes is the product of the GST-3 gene.

The variable level of staining activity of the GST-1 and GST-2 isozymes in identical tissues from different individuals was quite striking. These differences may reflect varying degrees of enzyme induction and synthesis. The glutathione S-transferases from rat liver have been shown to be highly inducible (Chasseaud, 1979) and it is possible that differential exposure of individuals to varying therapeutic regimes prior to death may have given rise to varying levels of induction and expression. Variations in the level of expression of GST-3 were also observed and may represent a similar response to induction. However, previous studies by Scott and Wright (1980) have suggested that erythrocyte glutathione S-transferase (GST-3) activities are under genetic control and the variation observed here could possibly reflect additional modifying genetic factors.

In the present experiments three additional forms of glutathione S-transferase were identified in several tissues. GST-4 was detected only in skeletal muscle and heart and GST-5 only in the brain. Furthermore every tissue examined except erythrocytes was found to contain GST-6.
Laisney et al. (1984) described an isozyme in muscle which they also termed GST-4. These authors reported the presence of GST-4 in other tissues including liver, kidney, lung, spleen, thymus and brain. However the isozyme termed GST-4 in the present study was observed only in skeletal muscle and heart. Laisney et al. (1984) reported an isozyme in brain with a similar electrophoretic mobility to the GST-5 isozyme observed in the present study, however the isozyme observed by Laisney et al. (1984) was also weakly present in lung tissue.

Since the GST-6 isozyme was not detected in erythrocytes in the present experiments, it seems unlikely that GST-6 is similar to one of the two forms reported in erythrocytes by Strange et al. (1983). These authors did not present any photographic evidence from other tissues, to allow direct comparisons, but they reported that the presence of two forms of GST-3 in other tissues were the result of post-synthetic modification of the product of the GST-3 locus. It is therefore possible that the isozyme termed GST-6 in the present study is identical to the more anodal form of GST-3 observed in tissue samples by Strange et al. (1983). Further studies are therefore required to determine if GST-6 is a modified form of GST-3 or is the product of a separate gene.

It is evident from the results obtained from the electrophoretic survey of glutathione S-transferase isozymes in human tissues and the comparison of these results with the
findings of Laisney et al. (1984) and Strange et al. (1984b), that there are several unanswered questions regarding the genetic relationship of the cationic GST-2 isozymes and the more recently detected isozymes (GST-4, GST-5 and GST-6) which have varying degrees of tissue specificity. It is possible that these isozymes could be the products of separate genes or could be the result of different combinations and modifications of subunits derived from the previously described genes. The experiments reported in the following chapters of this thesis have been designed to further investigate the relationship between the products of the three previously described loci and to evaluate the possibility that GST-4, GST-5 and GST-6 are the products of additional genes.
Chapter 3

The purification and biochemical characterization of the GST-1 isozymes from human liver

Human glutathione S-transferases were first purified in 1975 and several homologous glutathione transferase isozymes were designated as α, β, γ, and δ on the basis of their increasing isoelectric points (Yates et al., 1975; see Chapter 1). Study of a large number of human liver samples by an electrophoretic technique has suggested that the human glutathione S-transferases are the products of the three distinct functional genes: GST-1, GST-2, and GST-3 (Board, 1987a, b). The regulation of these genes, and hence these proteins, are expressed at different levels in different tissues in agreement with the recent findings of Strang et al. (1988) and Leivo et al. (1989). It is likely that the products of the GST-1, GST-2, and GST-3 genes proposed by Board (1987a, b) are the same as the previously described neuronal, basic, and acidic forms, respectively. However, comparative studies of these different isozymes have not yet been made. The products of the GST-1 locus described by Board (1981a) include two electrophoretically distinct forms, type 1 and type 2, distinguishable by monoclonal antibody GST-1A and GST-P, and a second form, GST-1P. However,
Human glutathione S-transferases were first purified in 1975 and several basic glutathione S-transferase isozymes were designated as α, β, γ, δ and ε on the basis of their increasing isoelectric points (Kamisaka et al., 1975). Since then several research groups have purified the enzyme from various human tissues and at the present stage three types of human glutathione S-transferase (basic, near-neutral and acidic forms) have been identified according to their isoelectric points (Mannervik, 1985; see Chapter 1).

Studies of a large number of human liver samples by an electrophoretic technique strongly suggested that the human glutathione S-transferases are the products of the three distinct autosomal loci, GST-1, GST-2 and GST-3 (Board, 1981a & b). The results of Chapter 2 show that these genes are expressed at different levels in different tissues in agreement with the recent findings of Strange et al. (1984b) and Laisney et al. (1984). It is likely that the products of the GST-1, GST-2 and GST-3 genes proposed by Board (1981 a & b) are the same as the previously described near-neutral, basic and acidic forms respectively, however, comparative studies of these different isozymes have not yet been made. The products of the GST-1 locus described by Board (1981a) include two electrophoretically distinct forms, type 1 and type 2, attributable to autosomal alleles, GST-1*1 and GST-1*2, and a common null allele, GST-1*0. Hetero-
zygotes express the type 1 and type 2 allele products and also produce a heterodimeric isozyme with an intermediate electrophoretic mobility. Board (1981a) originally reported the frequency of the three alleles in Australians to be
\[ \text{GST-1}^{*1} = 0.1061, \text{GST-1}^{*2} = 0.0784 \text{ and GST-1}^{*0} = 0.8154. \]

The frequency of these alleles in other populations was also reported (see Chapter 1).

Board (1981b) found the total activity of the glutathione S-transferases in the liver from individuals homozygous for a null allele at the GST-1 locus (GST-1*0/GST-1*0) to be significantly lower than that in a pooled group of individuals who expressed products of either the GST-1*1 or GST-1*2 alleles, and concluded that individuals homozygous for a null allele at the GST-1 locus may have a relatively greater risk than others in response to chemical carcinogens.

In the present experiments, the proposed allelic products of the GST-1 locus, GST-1*1 and GST-1*2 have been purified from human liver and characterized. The results support the proposal that the two isozymes are the products of a single locus as they both have similar properties that differ significantly from the products of other proposed loci.
MATERIALS AND METHODS

Materials: The substrates and inhibitors used were purchased from Sigma Chemical Company (St. Louis, MO, USA) except 1-chloro-2,4-dinitrobenzene (CDNB) which was obtained from Fluka AG (Switzerland). All other chemicals were analytical grade and some special materials were obtained from various companies which are indicated in the text.

Assay procedure for glutathione S-transferase:
Glutathione S-transferase activity was measured with slight modifications of the spectrophotometric method described by Habig et al. (1974b). A 1 ml reaction mixture containing 940 μl of 0.1 M sodium phosphate buffer (pH 7.5), 20 μl of 10 mM GSH, 20 μl of 10 mM CDNB (10.1 mg of CDNB was dissolved in 2 ml of ethanol at more than 30°C and then 3 ml of distilled water was added into the solution) and 20 μl of the sample was assayed at 37°C. The rate of increase in optical density at 340 nm was measured against that of a blank, where the sample was replaced by 0.1 M sodium phosphate buffer (pH 7.5). Reaction rates were normally recorded for 10-15 minutes in recording spectrophotometers (Varian SuperScan 3 or Gilford 2600). The 0.1 M sodium phosphate buffer (pH 7.5) and 10 mM CDNB were incubated at 37°C before the assay, however 10 mM GSH was kept on ice. A unit of enzyme activity was defined as the production of 1 μmole of the S-(2,4-dinitrophenyl)glutathione conjugate.
per minute and was calculated using a mM extinction coefficient of 9.6 (Habig et al., 1974). Specific activity is expressed as units (U)/mg of protein. The calculation of the enzyme activity using CDNB and GSH as substrates is described as follow:

\[
A = \frac{\Delta O.D._{\text{340}}}{9.6} \times \frac{1000 \mu l}{\text{sample volume (\mu l)}} \times \frac{1}{\text{protein (mg/ml)}}
\]

- \(A\): \(\mu\)mole conjugate/mg/minute = U/mg
- \(\Delta O.D._{\text{340}}\): change of optical density/minute
- 9.6: mM extinction coefficient of S-(2,4-dinitrophenyl)glutathione

**Protein determination:** Protein concentration was determined by the method of Bradford (1976) using coomassie brilliant blue G-250 (Sigma) because the commonly used method of Lowry et al. (1951) is strongly affected by some reductants such as reduced glutathione. Coomassie brilliant blue G-250 (100 mg) was dissolved in 50 ml of 95% ethanol and added to 100 ml of 85% orthophosphoric acid (\(\text{H}_3\text{PO}_4\)). The resulting solution was diluted to a final volume of one litre (Protein reagent). Five ml of the protein reagent was pipetted into test tubes. The sample (0.1 ml) was added to the tubes and the contents were mixed by vortexing. The absorbance at 595 nm was measured spectrophotometrically after 2 minutes and before one hour against a reagent blank prepared from 0.1 ml of H\(_2\)O and 5
ml protein reagent. The protein concentration was obtained by comparison with standard curves prepared using bovine serum albumin.

Starch gel electrophoresis: The electrophoresis and specific glutathione S-transferase staining were performed by the method of Board (1980). The detailed procedures are described in Chapter 2.

SDS/polyacrylamide gel electrophoresis: Vertical SDS polyacrylamide electrophoresis was carried out by the method of Laemmli (1970). A 12.5%(w/v) running gel was prepared by combination of the following components:

i) 15 ml of the acrylamide solution (29.2%(w/v) acrylamide and 0.8%(w/v) N,N'-methylene-bis acrylamide (Bis))
ii) 9 ml of the running gel buffer (1.5 M Tris and 0.4%(w/v) sodium dodecyl sulfate (SDS), pH8.8)
iii) 12 ml of distilled water
iv) 150 µl of 10% (w/v) ammonium persulfate ((NH₄)$_2$S₂O₈)
v) 45 µl of N,N,N',N'-tetramethyl-ethylenediamine (TEMED).

After deaeration briefly under reduced pressure, approximately 13 ml of the solution was poured into a slab-gel mould (13.5 cm long x 12 cm wide x 1 mm thick). Distilled water was carefully poured on the top of the running gel to provide a level gel top and to prevent contact with O₂. After polymerization the gel was overlayed with a stacking gel containing:
i) 3.0 ml of the acrylamide solution
ii) 4.0 ml of the stacking gel buffer (0.05 M Tris/0.4% (w/v) SDS, pH6.8)
iii) 9.6 ml of distilled water
iv) 60 µl of 10%(w/v) ammonium persulfate
v) 30 µl of TEMED.

The electrode buffer contained:
  i) 0.025 M Tris
  ii) 0.192 M glycine
  iii) 1%(w/v) SDS and was adjusted to pH8.3.

The sample buffer was the mixture of
  i) 10%(w/v)glycerol
  ii) 5%(w/v) β-mercaptoethanol
  iii) 2.3%(w/v) SDS.

An equal volume of the sample and the sample buffer were kept at 100°C for approximately 4 minutes. After cooling, 5 µl of bromophenol blue solution (0.05%(w/v) bromophenol blue and 70%(w/v) glycerol in 0.0625 M Tris (pH6.8)) was added into 0.1 ml of the mixture. Electrophoresis was performed at room temperature with a constant current of 10 mA while the bromophenol blue was running through the stacking gel. When the bromophenol blue entered the running gel, the current was increased to 25-30 mA. After electrophoresis the gel was fixed in methanol/acetic acid/H₂O (5:1:4) for 20-30 minutes at room temperature, and then it was stained in a solution of 0.1%(w/v) coomassie brilliant blue R in methanol/acetic acid/H₂O (1:1:8) at 60°C for 1-2 hours, and then destained in a solution of methanol/acetic acid/H₂O (1:1:8) at 60°C until the background of the gel was clear.

Molecular weight determination: Molecular weights were estimated by comparison of the relative electrophoretic mobility of the sample protein with a standard curve derived from a plot of the relative electrophoretic mobility against log₁₀ MW of standard proteins.
Purification of GST-1*1 and GST-1*2 isozymes from human liver:

Step 1. Human livers were obtained and stored at -20°C until the purification of glutathione S-transferase was carried out. Livers expressing either the GST-1 type 1 or type 2 allelic products were selected after starch gel electrophoresis and specific GST staining of small samples. Homogenates of 89.6 g of a liver containing GST-1 type 1 and 85.5 g of a liver containing GST-1 type 2 were made in four volumes of 10 mM Tris/HCl buffer, pH 8.0 (Tris/HCl buffer) respectively. The homogenates were centrifuged at 10,000 x g for 2 hours at 4°C and the supernatants were filtered through plugs of cotton, and 358 ml of the supernatant for GST-1 type 1 and 342 ml of that for GST-1 type 2 were obtained. Ammonium sulfate was added to both supernatants to 40% saturation. The mixtures were stirred overnight at 4°C and then centrifuged for 30 minutes at 10,000 x g at 4°C. The precipitates were dissolved in 50 ml of the Tris/HCl buffer and dialyzed against five changes of 5 litres of the Tris/HCl buffer for 24 hours.

Step 2. The dialyzed samples (110 ml for type 1 and 115 ml for type 2) were passed through columns of DEAE cellulose (Whatman DE52) (3.5 cm x 30 cm). After the columns were extensively washed with Tris/HCl buffer (pH 8.0) and protein (O.D.280) was no longer eluted, a 2 litre KCl gradient from 0 to 0.3 M KCl in Tris/HCl buffer was used for elution of GST-1*1 and GST-1*2. Fractions of 8 ml were
collected at a flow rate of approximately 70 ml/h. Those with GST activity were checked by starch gel electrophoresis to confirm the presence of the GST-1*1 and GST-1*2 isoenzymes.

Step 3. Ammonium sulfate was added to each of the pooled GST-1*1 or GST-1*2 samples to a final concentration of 40% saturation. The mixtures were gently stirred overnight at 4°C and then centrifuged for 30 minutes at 10,000 x g at 4°C. The precipitate was dissolved in 20 ml of 10 mM sodium phosphate buffer (pH 7.0) and dialyzed against five changes of 5 litres of 10 mM sodium phosphate buffer (pH 7.0). The dialyzed samples (55 ml for GST-1*1 and 40 ml for GST-1*2) were applied to columns of Affi-Gel Blue (BIORAD) (2.3 cm x 12 cm) which were equilibrated with 20 mM sodium phosphate buffer (pH 7.0). The columns were washed with 20 mM sodium phosphate buffer (pH 7.0) until the eluant was free of protein. A solution of 2 mM GSH adjusted to pH 7.0 was used for the elution of GST-1*1 and GST-1*2. After the main peak of the activity was eluted, the concentration of GSH was increased up to 20 mM (pH 7.0), however no further GST-1*1 or GST-1*2 were detectable in the eluant.

The purified samples were kept in the presence of 30%-40% of glycerol at -20°C until they were used.

Substrate specificity: Specific activities of the GST-1*1 and GST-1*2 isoenzymes were measured with several compounds which have been previously reported to be sub-
strates for rat glutathione S-transferases (Habig et al., 1974b). In addition to CDNB, 1,2-dichloro-4-nitrobenzene (DCNB), 1,2-epoxy-3-(p-nitrophenoxy)propane (ENPP), bromosulfophthalein (BSP), \textit{trans}-4-phenyl-3-buten-2-one (tPB), and ethacrynic acid (EA) were used as substrates. A 1 ml reaction mixture contained 940 µl of 100 mM sodium phosphate buffer (pH 7.5), 20 µl of sample, 20 µl of GSH and 20 µl of a substrate was assayed at 37°C at an appropriate wave length. The concentration of each substrate used and the wave length for each spectrophotometric assay are shown in Table III-1.

Table III-1. Substrate concentrations and wave length for glutathione S-transferase assays.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>GSH</th>
<th>Wave length</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNB (50 mM) b</td>
<td>50 mM</td>
<td>340 nm</td>
</tr>
<tr>
<td>DCNB (50 mM) b</td>
<td>50 mM</td>
<td>345 nm</td>
</tr>
<tr>
<td>ENPP (25 mM) b</td>
<td>250 mM</td>
<td>360 nm</td>
</tr>
<tr>
<td>BSP (10 mM) c</td>
<td>250 mM</td>
<td>330 nm</td>
</tr>
<tr>
<td>tPB (2.5 mM) b</td>
<td>12.5 mM</td>
<td>290 nm</td>
</tr>
<tr>
<td>EA (10 mM) d</td>
<td>12.5 mM</td>
<td>270 nm</td>
</tr>
</tbody>
</table>

\(^a\): 20 µl of these solutions were used for each assay
\(^b\): CDNB, DCNP, ENPP and tPB were dissolved in ethanol
\(^c\): BSP was dissolved in distilled water
\(^d\): EA was dissolved in chloroform
The change in optical density was recorded against that of a blank, where the sample was replaced by 0.1 M sodium phosphate buffer (pH7.5). Reactions were normally recorded for approximately 40-60 minutes with the exception of CDNB which was recorded for 10-15 minutes.

Kinetic analysis and inhibition studies: The $K_m$ values of GST-1*1 and GST-1*2 were determined using different concentrations of the substrates, GSH and CDNB (0.08 mM to 0.4 mM) in sodium phosphate buffer (pH7.5) at 37°C. The data were plotted by the Lineweaver-Burk method ($1/v$ against $1/[\text{CDNB}]$ or $1/[\text{GSH}]$). The $K_m$ value was calculated by a linear regression of each plot.

Glutathione peroxidase activity of the purified samples was measured with slight modifications of the spectrophotometric method described by Beutler (1974). A mixture of 750 µl of 0.1 M potassium phosphate buffer (pH7.0), 20 µl of 100 mM GSH, 100 µl of 100 U/ml glutathione reductase (Boehringer Manheim GmbH, West Germany), 100 µl of 2 mM NADPH (Sigma) and 20 µl of the sample were preincubated at 37°C for 10 minutes. The substrate, either 10 µl of 7 mM t-butyl hydroperoxide, 1.2 mM cumen hydroperoxide, or 1.25 mM hydrogen peroxide was then added to the assay system. The activity at 37°C was measured spectrophotometrically by the determining the rate of conversion of NADPH to NADP at 340 nm against that of the blanks without either a peroxide substrate or an enzyme sample.
The effect of cholate, chenodeoxycholate, lithocholate 3-sulfate, ellagic acid and bromosulfophthalein on GST-1*1 and GST-1*2 activities were examined in the CDNB assay system described previously. The final concentrations of these inhibitors were 0.5 µM, 2 µM, 10 µM, 50 µM, 100 µM and 200 µM. Chenodeoxycholate, lithocholate 3-sulfate and ellagic acid were dissolved in ethanol and cholate in distilled water. However, the final concentration of ethanol in the assay system did not exceed 2%.

**Heat stability:** First, the purified samples were incubated at 45°C for 10, 20 and 30 minutes. After incubation each sample was placed on ice before the glutathione S-transferase activity was determined. Secondly, the samples were incubated at different temperatures (30, 40, 50 and 60°C) for 10 minutes. After incubation, each sample was placed on ice before the glutathione S-transferase activity was measured.

**pH optimum:** The pH optima of GST-1*1 and GST-1*2 were determined by the use of the Tris/glycine/phosphate buffers described by Beutler et al. (1968) and using CDNB and GSH as substrates. The pH range studied extended from pH 5.5 to pH 11.5 in 0.5 steps. The standard buffers were prepared as follows. The stock solution contained 12.1 g of Tris, 7.5 g of glycine, and 13.8 g of NaH₂PO₄(H₂O) in 1 litre of water. Aliquots of the stock solution (50 ml) were adjusted
to the required pH with NaOH or HCl. The volume of each buffer was then brought to 75 ml and was stored frozen at -20°C in 10 ml aliquots. When the experiments using these buffers were carried out, the buffers were brought to 37°C and the pH again checked and adjusted.

Before the purified enzymes were used in the present experiments, the sample was dialyzed against 5 litres of 20 mM phosphate buffer (pH 7.5) and then activity of the sample in the standard assay system (pH 7.5) as a control was diluted to approximately 0.1-0.2 µM/min./ml.

**Immunological studies:**

**Preparation of antiserum:** Equal volumes of the purified enzyme (~50 µg) and Freund's adjuvant (CSL, Australia) were vigorously mixed to give a stable emulsion. The antigens were subcutaneously injected at more than five sites on the back of young adult rabbits. The rabbits were immunized four times at 7-10 day intervals. Freund's complete adjuvant was used for the first injection and Freund's incomplete adjuvant was used for all subsequent injections. Seven days after the last injection, 20-30 ml of blood was obtained from the marginal ear vein and was allowed to clot overnight at room temperature. The serum was decanted from the clot and centrifuged to remove traces of fibrin and erythrocytes. The antiserum with 0.1% sodium azide (NaN₃) was stored in 2 ml aliquots at -20°C until used.

**Immunodiffusion:** Microscope slides were coated with 1% (w/v) agarose in phosphate buffered saline (pH 7.0). Wells
were punched according to the pattern required. Immunodiffusion was carried out overnight at room temperature in a moist atmosphere. Immunoprecipitates were visible as white precipitin lines in the gel when viewed against a dark background. However, for increased sensitivity, the gel was stained for protein. The glass slide was covered with a wet filter paper (Whatman 3MM) and was squashed under a 1 Kg weight for 15 minutes. The slide was then soaked in phosphate buffered saline for several hours to remove serum protein, and then dried and stained in 0.2% coomassie brilliant blue R (Sigma) in methanol/acetic acid/H$_2$O (9:2:9) for 3-5 minutes. The slide was destained in Tepol solution (Tepol/acetic acid/H$_2$O = 2.5:2.5:95) and then rinsed under running tap water. Finally the stained gel was dried.

RESULTS

Purification of GST-1*1 and GST-1*2: Tables III-2 and III-3 show the results of the purification steps. The separation patterns of the enzyme by DEAE cellulose and Affi-Gel Blue chromatography are also shown in Figures III-1A & B for GST-1*1 and III-2A & B for GST-1*2. The final yields were 3.3% for GST-1*1 and 4.9% for GST-1*2. Although the yields were low, the purity of the sample was very high, as judged by SDS/polyacrylamide gel electrophoresis (Figure III-3). The two isozymes were identified by starch gel electrophoresis at each purification step.
### Table III-1. Purification of GST-1*1 from human liver

<table>
<thead>
<tr>
<th></th>
<th>Total volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Activity (U/ml)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>358</td>
<td>14248</td>
<td>0.507</td>
<td>1815.06</td>
<td>0.127</td>
<td>100</td>
</tr>
<tr>
<td>DEAE cellulose c</td>
<td>185</td>
<td>2505</td>
<td>1.80</td>
<td>347.08</td>
<td>0.139</td>
<td>19.2</td>
</tr>
<tr>
<td>Affi-Gel Blue d</td>
<td>220</td>
<td>6.4</td>
<td>0.27</td>
<td>59.4</td>
<td>9.281</td>
<td>3.3</td>
</tr>
</tbody>
</table>

### Table III-2. Purification of GST-1*2 from human liver

<table>
<thead>
<tr>
<th></th>
<th>Total volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Activity (U/ml)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>342</td>
<td>13133</td>
<td>2.30</td>
<td>7866.0</td>
<td>0.06</td>
<td>100</td>
</tr>
<tr>
<td>DEAE cellulose c</td>
<td>425</td>
<td>2048</td>
<td>0.34</td>
<td>144.50</td>
<td>0.071</td>
<td>18.4</td>
</tr>
<tr>
<td>Affi-Gel Blue d</td>
<td>82</td>
<td>4.0</td>
<td>0.47</td>
<td>38.54</td>
<td>9.635</td>
<td>4.9</td>
</tr>
</tbody>
</table>

a: Protein concentration was determined by the method of Bradford (1976).
b: The enzyme activity was measured at 37°C and pH7.5 using 0.2 mM CDNB and 0.2 mM GSH as substrates. U = µM conjugate/minute.
c: The chromatography was performed at pH8.0 and a KCl gradient was used to elute the enzyme.
d: Affi-Gel Blue (BIORAD) was used at pH7.0 and the enzyme was eluted by 2 mM GSH (pH7.0).
Figure III-1A. Elution pattern of GST-1*1 and protein from DEAE cellulose (pH8.0).

Figure III-1B. Elution pattern of GST-1*1 and protein from Affi-Gel Blue (pH7.0).
Figure III-2A. Elution pattern of GST-1*2 and protein from DEAE cellulose (pH8.0).

Figure III-2B. Elution pattern of GST-1*2 and protein from Affi-Gel Blue (pH7.0).
Molecular weight: The subunit molecular weight was determined by SDS/polyacrylamide gel electrophoresis and the sizes of the subunits were 27,500 for both GST-1*1 and GST-1*2 (Figure III-3).

Kinetic analysis, substrate specificity and inhibition studies: The affinity of both allelic products for substrates such as CDNB and GSH appear to be very similar. The $K_m$ values for both substrates are shown in Table III-4.

Table III-4. The $K_m$ values (mM) for CDNB and GSH

<table>
<thead>
<tr>
<th></th>
<th>GST-1*1</th>
<th>GST-1*2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNB</td>
<td>0.46</td>
<td>0.46</td>
</tr>
<tr>
<td>GSH</td>
<td>0.75</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Neither isozyme exhibited any glutathione peroxidase activity with the three different peroxide substrates. In addition, neither isozyme gave measurable levels of activity with any of the other substrates tested. The inhibitory effect of five chemicals are shown in Figure III-4. Inhibition profiles of GST-1*1 and GST-1*2 isozymes were similar in the experiments using chenodeoxycholate, lithocholate 3-sulfate and ellagic acid. The GST-1*1 isozyme appears to be more sensitive to inhibition by cholate and bromosulphophthalein than the GST-1*2 isozyme, however the trends in both inhibition profiles were similar.
Figure III-3. SDS/polyacrylamide gel electrophoresis of purified GST-1*1 and GST-1*2

Lane 1: Molecular weight markers
Lane 2: GST-1*1
Lane 3: GST-1*2
Figure III-4. The effect of some inhibitors on GST-1*1 and GST-1*2 activities.

A: cholate
B: chenodeoxycholate
C: lithocholate 3-sulfate
D: ellagic acid
E: bromosulfophthalein

GST-1*1 (●-●)
GST-1*2 (○-----○)
Heat stability: The relative stability of GST-1*1 and GST-1*2 isozymes at 45°C are shown in Figure III-5A and the effects of different temperatures on GST-1*1 and GST-1*2 are shown in Figure III-5B. It is evident that both isozymes have similar heat stability profiles.

pH optimum: The variations of GST-1*1 and GST-1*2 activities with pH are shown in Figure III-6. The spontaneous reaction between GSH and CDNB without the enzyme is also shown in Figure III-6. The pH curve of the GST-1*2 isozyme appeared to be shifted to a slightly higher pH than that of GST-1*1, and the pH with maximum activity was pH 7.5 for GST-1*1 and pH 8.0 for GST-1*2.

Immunological studies: Double immunodiffusion of GST-1*1 and GST-1*2 and antiserum raised against GST-1*2 is shown in Figure III-7. These results show a reaction of identity between these two proteins.

DISCUSSION

Because the two livers used for the purification produced only the product of the GST-1*1 allele or GST-1*2 allele, the final purified material from these livers was not cross contaminated. Although the products of the GST-2 locus were found in both livers, they were removed from each sample by their passage through DEAE cellulose at the first step. The product of the GST-3 locus (GST-3 isozyme),
Figure III-5. The effect of temperature on GST-1*1 and GST-1*2.
A: incubated for 10 minutes at various temperatures.
B: incubated for various times at 45°C.

GST-1*1 (●——●)
GST-1*2 (○——○——○)

Figure III-6. The effect of pH on GST-1*1 and GST-1*2 activities.

GST-1*1 (●——●)
GST-1*2 (○——○——○)

Figure III-7. Double immunodiffusion of GST-1*1 and GST-1*2 against anti GST-1*2.
a minor component in liver, was separated from the GST-1 product by DEAE cellulose chromatography with a KCl gradient and Affi-Gel Blue at both the first and second steps. Affi-Gel Blue is Cibacron blue (Ciba Geigy Co.) attached to 5% crosslinked agarose. In the present experiments, the application of this medium for the purification of glutathione S-transferases has been shown for the first time. The affinity of the glutathione S-transferases for this medium was found to be considerably dependent on the pH of the eluting buffer and some glutathione S-transferase isozymes can be selectively eluted by the application of different concentrations of GSH. This suggests that there may be some specific interaction between Cibacron blue and the active site. The recent report of Tahir et al. (1985) that Cibacron blue can inhibit the glutathione S-transferase activity also supports this conclusion.

The data obtained in the present studies indicate that the catalytic properties of the GST-1*1 and GST-1*2 isozymes are very similar. Both isozymes were found to have similar affinities for CDNB and GSH. Neither isozyme exhibited glutathione peroxidase activity which has been reported in some cationic rat and human liver glutathione S-transferases (Prohaska and Ganther, 1977; Awasthi et al., 1980). Several different compounds including DCNB, ENPP, BSP, tPB and EA, which have previously been shown to be substrates for different rat glutathione S-transferases (Habig et al., 1974b), did not appear to be substrates for
either the GST-1*1 or GST-1*2 isozymes. However, the rate of reaction of these substrates with rat liver glutathione S-transferases was originally reported to be very low, it is therefore possible that the rate of reaction catalyzed by the GST-1 isozymes is below the limit of detection in the present studies.

The inhibitors used in the present experiments have been previously reported by several research groups. One of the inhibitors reported early in research on the glutathione S-transferases, bromosulfophthalein, was used as an inhibitor of the enzyme from grass grubs and also sheep liver (Clark et al., 1967). More recently, bile acids (cholate, Chenodeoxycholate and lithocholate 3-sulfate) were reported to inhibit the activity of all the basic and neutral glutathione S-transferases of rat liver, but marked differences in the effects of bile acids on individual rat isozymes have been reported by Hayes and Chalmers (1983). Ellagic acid, a commonly occurring plant phenol, was also reported to be a potent in vitro inhibitor of glutathione S-transferases from rat liver (Das et al., 1984). It is clear from the present studies that, although these compounds inhibit the GST-1 isozymes, there is little difference in their affects on the two forms of GST-1.

The products of alleles at a single locus often differ only by a single amino acid substitution, and their functional properties are often similar unless the substitution destabilizes the isozymes tertiary structure or affects the
active site. The present data clearly show the similarities in subunit molecular weight, substrate affinity, heat stability, pH optimum and susceptibility to inhibition of GST-1*1 and GST-1*2 isozymes. These data, therefore, support the proposal that both GST-1*1 and GST-1*2 isozymes are the allelic products of the GST-1 locus in man. In addition the reaction of identity between GST-1*1 and GST-1*2 isozymes and anti GST-1*2 serum also supports the conclusion that these two isozymes are allelic variants.

Warholm et al. (1981b & 1983) have reported the properties of an isozyme termed glutathione S-transferase µ purified from human liver. Their data show that glutathione S-transferase µ has an isoelectric point of 6.6, a high specific activity for trans-4-phenyl-3-buten-2-one (tPB), benzo[a]pyrene-4,5-oxide and styrene-7,8-oxide, $K_m$ (GSH) of 0.16 and $K_m$ (CDNB) of 0.65, pH optimum of 7.5 and subunit molecular weight of 26,300. This isozyme has been classified under the category of near-neutral glutathione S-transferase by Mannervik (1985).

Comparison of the GST-1 isozymes with glutathione S-transferase µ reveals several similarities as well as differences. Although the isoelectric points of the GST-1 isozymes were not determined, their migration on a starch gel at pH8.6 was the slowest of all the anodal isozymes found in liver and suggests their pl is around pH7. This is in agreement with the pl of 6.6 reported for glutathione S-transferase µ. Similarly, the subunit molecular weight of
glutathione S-transferase µ was reported to be 26,300 which compares with the value of 27,500 obtained for the GST-1 isozymes studied here. The difference in these values is probably not significant when the probable differences in standard proteins and electrophoretic procedures between laboratories are considered. One notable difference between the reported characteristics of glutathione S-transferase µ and the GST-1 isozymes is the apparently low activity of the GST-1 isozymes with tPB as a substrate.

The report of Warholm et al. (1983) that glutathione S-transferase µ could only be detected in the livers of 60% of subjects is of particular significance. Board (1981a) and Strange et al. (1984b) have reported that there is a common null allele at the GST-1 locus in the Australian and British populations which occurs with a frequency of .54 and .59 respectively. Since no other isozymes from liver appear to be deficient at that frequency, and given the other similarities, it is highly likely that the glutathione S-transferase µ reported by Warholm et al. (1983) is identical to one of the GST-1 isozymes originally described by Board (1981a) and characterized in more detail here.
Chapter 4

The purification and biochemical characterization of GST-2 isozymes from human liver
INTRODUCTION

The major glutathione S-transferases expressed in the liver are the cationic forms (see Chapter 2). Board (1981a) suggested that the cationic glutathione S-transferase isozymes from human liver were the products of a single genetic locus termed GST-2. Differences observed in the distribution of the cationic isozyme bands between different individuals led to the suggestion that the GST-2 locus was polymorphic with two alleles (GST-2*1 and GST-2*2) coding for isozyme subunits with differing electrophoretic mobilities (Board, 1981b). It was suggested that the most cathodal isozyme was a homodimer of the type 1 subunit, the isozyme nearest the origin was a homodimer of the type 2 subunit, and the intermediate isozyme was a heterodimer of both the type 1 and type 2 subunits. In the following discussion the type 1 homodimer will be termed 'GST-2*1' and the type 2 homodimer will be termed 'GST-2*2'.

More recently Strange et al. (1984b) and Laisney et al. (1984) have shown that the GST-2 phenotype was not a constant individual characteristic and varied between tissues within individuals. Their observations led to the suggestion that the various forms of GST-2 found in the liver and other tissues are the result of post-translational modification of a single gene product (Strange et al., 1984b). As mentioned in Chapter 1, Kamisaka et al. (1975) purified several cationic glutathione S-transferase isozymes from
human liver with isoelectric points ranging from 8-10. These authors found that the biochemical and immunological characteristics of the cationic glutathione S-transferases were very similar and also concluded that they are the result of post-translational modification. In contrast Stockman et al. (1985) have recently reported the purification and characterization of two human liver cationic glutathione S-transferases which are composed of two immunologically different subunits. Stockman et al. (1985) suggest that there are two homodimeric isozymes containing either two $B_1$ or two $B_2$ subunits in addition to a heterodimeric isozyme containing both the $B_1$ and $B_2$ subunits.

The structural relationship proposed by Stockman et al. (1985) is very similar to that originally proposed by Board (1981a) and it seems likely that the $B_1$ and $B_2$ subunits are equivalent to the proposed GST-2 type 1 and GST-2 type 2 allelic products. However, if $B_1$ and $B_2$ are immunologically distinct as shown by Stockman et al. (1985), then it is more likely that they are the products of different gene loci rather than the products of alleles at the same locus.

The present experiments were designed to determine some of the biochemical and immunological characteristics of the GST-2 isozymes originally identified electrophoretically by Board (1981a) in order to relate these to the isozymes characterized by others, and to provide further information that may allow a clearer understanding of the
possible genetic relationships between the different cationic glutathione S-transferases and their overall relationships with the more acidic forms.

MATERIALS AND METHODS

Materials, and the detailed procedures for the determination of glutathione S-transferase activity, protein concentration, starch gel electrophoresis, and SDS/polyacrylamide gel electrophoresis have been described in Chapters 2 and 3.

Purification of GST-2*1 isozyme from human liver:

Step 1. A human liver, where both GST-2*1 and GST-2*2 isozymes and GST-1 isozymes were present, was used for the purification of the GST-2*1 isozyme. A homogenate of the liver (396 g) was made in four volumes of 10 mM Tris/HCl buffer (pH 8.0) by an electric blender and a motor-driven teflon-glass homogenizer. The homogenate was centrifuged at 10,000 x g for 2 hours and lipids were removed by passage through a plug of cotton. The extract had a final volume of 1600 ml. The extract was passed through a column of DEAE cellulose (Whatman DE52) (3.5 cm x 30 cm) pre-equilibrated at pH 8.0 with 10 mM Tris/HCl buffer. The active fractions that passed straight through the column contained GST-2 isozymes and had a total pooled volume of 510 ml.
Step 2. Solid ammonium sulfate was added to the pooled eluant from the DEAE cellulose to 40% saturation and stirred at 4°C overnight. The mixture was centrifuged at 10,000 x g for 30 minutes, and the precipitate was collected. The precipitate was dissolved with 50 ml of 10 mM phosphate buffer (pH6.7) and the mixture was dialyzed against five changes of 5 litres of 10 mM phosphate buffer (pH6.7). A column of CM cellulose (Whatman CM52) (3.5 cm x 30 cm) was prepared and pre-equilibrated at pH6.7 with 10 mM phosphate buffer and 75 ml of the dialyzed sample was applied to the column. The flow rate was 50 ml/h. The column was washed extensively with 10 mM phosphate buffer until glutathione S-transferase activity and protein (O.D.\textsubscript{280}) were no longer detected in the eluant. A 2-litre gradient from 0 to 150 mM KCl in 10 mM phosphate buffer was used to elute the GST-2*1 isozyme from the column. After chromatography the glutathione S-transferase isozymes were identified in the active fractions, three pooled fractions were obtained, Pool I from fractions 15-36, Pool II from fractions 96-110, Pool III from fractions 111-126. Pool I contained both GST-1 and GST-2*2 isozymes. The intermediate band between GST-2*1 and GST-2*2 on starch gel after electrophoresis was included in Pool II. Pool III contained mainly the GST-2*1 isozyme and its final volume was 185 ml.

Step 3. The pooled sample was concentrated by ultrafiltration through an Amicon DIAFLO PM10 membrane and
dialyzed against five changes of 5 liters of 10 mM Tris/HCl buffer (pH8.0). The sample was applied to the column of Affi-Gel Blue (BIORAD) (pH8.0) (2.3 cm x 12 cm). After glutathione S-transferase activity and protein were no longer eluted from the column, several steps of GSH (2-40 mM) adjusted at pH8.0 were used to elute GST-2*1. The active fractions were pooled in three groups: pool I from fractions 191 to 207 (71 ml), Pool II from fractions 209 to 237 (120 ml), and Pool III from fractions 239 to 261 (86 ml). Starch gel electrophoresis and specific glutathione S-transferase staining showed that Pool III contained the GST-2*1 isozyme.

Step 4. The sample was again concentrated by ultrafiltration and dialyzed against 20 mM phosphate buffer (pH7.0). The sample (15 ml) was applied to a column of GSH-Agarose (pH7.0) (2.3 cm x 6 cm). The column was washed extensively with 20 mM phosphate buffer (pH7.0) until protein was no longer detectable in the eluant and then 50 mM Tris (pH9.6) containing 5 mM GSH was applied to elute GST-2*1 from the column. The pooled volume of the active eluant was 22 ml.

Purification of GST-2*2 isozyme from human liver:

Step 1. The GST-2*2 isozyme was purified from a different liver sample to that which was used to purify the GST-2*1 isozyme. A homogenate of liver (218.9 g), which expressed all GST-2 isozymes and the product of the GST-1*1
allele, was made in four volumes of 10 mM Tris/HCl buffer (pH 8.0) by the use of a blender and a motor-driven teflon-glass homogenizer. The homogenate was centrifuged at 10,000 x g for 2 hours and the supernatant was filtered through a plug of cotton. A total extract volume of 770 ml was obtained. The extract was concentrated by the addition of ammonium sulfate to 40% saturation and the precipitate was obtained by centrifugation at 10,000 x g for 30 minutes. The precipitate was dissolved in 150 ml of 10 mM phosphate buffer (pH 6.7) and dialyzed against five changes of 5 litres of 10 mM phosphate buffer (pH 6.7). The dialyzed sample (275 ml) was divided equally and applied to two columns of DEAE cellulose (pH 6.7) (3.5 cm x 30 cm). At pH 6.7 the GST-2 isozymes were not retained by the DEAE cellulose column and the active fractions that passed straight through the columns were pooled (2,350 ml) and concentrated by precipitation at 40% saturation with ammonium sulfate. The precipitate was dissolved in 10 mM Tris/HCl (pH 7.5) and dialyzed against five changes of 5 litres of 10 mM Tris/HCl buffer (pH 7.5).

Step 2. The dialyzed sample was again divided equally and charged onto two columns of DEAE cellulose equilibrated at pH 7.5 with 10 mM Tris/HCl buffer (pH 7.5). The columns were washed extensively with 10 mM Tris/HCl buffer (pH 7.5) until protein was no longer detectable in the eluant. A 4 litre gradient from 0 to 0.3 M KCl in 10 mM Tris/HCl buffer (pH 7.5) was used to elute the enzyme from each column. Two
peaks of activity were found in the eluted fractions. The first peak which emerged before the application of the KCl gradient, contained GST-2. The second peak of activity eluted by the KCl gradient contained GST-1. Starch gel electrophoresis was used to detect fractions containing the GST-2*2 isozyme. The pooled volume of these fractions was 910 ml.

Step 3. Solid ammonium sulfate was added to the pooled sample of GST-2*2 rich fractions to give 40% saturation. The mixture was gently stirred for 8 hours at 4°C and centrifuged at 10,000 x g for 30 minutes. The precipitate was dissolved with 10 mM Tris/HCl (pH8.0) and dialyzed against five changes of 5 litres of 10 mM Tris/HCl (pH8.0). The dialyzed sample was applied to a column of CM-Sephadex (Pharmacia)(3.5 cm x 30 cm). The column was washed extensively until no further protein was eluted. A KCl gradient (0-0.3 M) was applied to elute GST-2 from the column. The GST-2*2 isozyme was again detected by starch gel electrophoresis and active fractions were pooled (900 ml). The sample was concentrated by ultrafiltration and dialyzed against 10 mM Tris/HCl (pH8.0).

Step 4. The sample (37 ml) was applied to a column of Affi-Gel Blue (BIO-RAD) (pH8.0) (2.3 cm x 12 cm). After the gel was extensively washed with Tris/HCl (pH8.0) until no further protein was eluted. The column was then developed by the addition of 2 mM GSH (pH8.0) and then 5 mM GSH (pH8.0) was added to completely elute the GST-2*2 isozyme.
from the column. The active fractions were pooled and with of final volume of 1,380 ml. The sample was concentrated by ultrafiltration.

Step 5. The concentrated sample (41 ml) was dialyzed against two changes of 5 litres of 20 mM phosphate buffer (pH7.0). The sample was divided into two equal volumes and applied to columns of GSH-agarose (Sigma) (pH7.0) (2.3 cm x 6 cm). After washing extensively with 20 mM phosphate buffer, pH7.0, pure GST-2*2 isozyme was eluted with 50 mM Tris containing 5 mM GSH (pH9.6). The active fractions were pooled to a final volume of 235 ml and concentrated by ultrafiltration.

The detailed procedures for evaluating substrate specificity, kinetic analysis, inhibition studies, heat stability, and pH optimum and immunodiffusion have been described in Chapter 3.

**Immunoblotting:** SDS/polyacrylamide gel electrophoresis was performed according to Laemmli (1970) as described in detail in Chapter 3. Electrophoretic transfer of proteins to nitrocellulose sheets and staining were carried out by modification of the methods described by Towbin et al. (1979) and Board (1984).
After running the electrophoresis, a nitrocellulose filter saturated in transfer buffer was placed on top of the gel. All air bubbles were pushed out by rolling a glass test tube over the surface. The gel and nitrocellulose filter were placed between sheets of pre-wetted 3MM chromatography paper and covered on each side by a wetted sponge and a porous plastic frame. The complete sandwich was held together by several rubber bands.

The transfer buffer was composed of 20 mM Tris, 150 mM glycine and 20% methanol (pH 8.3). Before using the transfer buffer, it was de-gassed for 30 minutes at reduced pressure. The electro-blot was carried out for more than 4 hours at 150 mA and 30 V at room temperature. After electro-blotting, the nitrocellulose filter was washed with 5% BLOTTO (5% milk powder in 50 mM Tris/150 mM NaCl buffer (pH 7.5) for 1 hour. This step blocked any remaining protein binding sites. The filter was soaked in 5% BLOTTO containing the first antibody, 10 µl/ml rabbit anti GST-1*2 in a small plastic bag. After 30 minutes exposure to the first antibody, the filter was washed extensively with 5% BLOTTO (3 x 100 ml x 5 minutes) on a rocking platform. After washing, a second antibody (goat anti rabbit IgG conjugated to alkaline phosphatase (Sigma), 1 µl/ml of 2% BLOTTO) was applied to the nitrocellulose filter in a small plastic bag for one hour at room temperature. After removal of the second antibody, the nitrocellulose filter was washed with several changes of 2% BLOTTO and finally with Tris/NaCl buffer (pH 7.5).
The filter was soaked in a staining solution containing 50 ml of staining buffer (3.7 g boric acid and 1.8 g NaOH in 1 litre) and 60 mg MgSO\(_4\)·7H\(_2\)O, 25 mg \(\beta\)-naphthylacid phosphate (Sigma), and 25 mg Fast Blue BB salt (Sigma). Zones of activity were normally evident within 10 minutes at room temperature.

RESULTS

Purification of GST-2*1 and GST-2*2 isozymes: The summary of the purification is shown in Table IV-1 for GST-2*1 and Table IV-2 for GST-2*2. The elution patterns for the purification of GST-2*1 are shown in Figures IV-1, 2 and 3 and those of GST-2*2 are shown in Figures IV-4, 5, 6, 7 & 8. GST-2*1 was separated from other glutathione S-transferase isozymes by CM cellulose (pH6.7) and GST-2*2 was partially separated from the GST-2*1 and GST-1 isozymes by several steps preceding the Affi-Gel Blue column and finally GST-2*2 was separated from GST-2*1 by chromatography on Affi-Gel Blue (pH8.0) because GST-2*1 appears to have a high affinity for that gel, as described in Step 3 of GST-2*1 purification procedure. Figure IV-9 shows the purified GST-2*1 and GST-2*2 isozymes on starch gel after electrophoresis and histochemical staining. Both isozymes appeared to be pure as judged by SDS/polyacrylamide gel electrophoresis (Figure IV-10).
Table IV-1. Purification of GST-2*1 from human liver

<table>
<thead>
<tr>
<th></th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Activity (U/ml)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1600</td>
<td>19600</td>
<td>1.82</td>
<td>2912</td>
<td>0.149</td>
<td>100</td>
</tr>
<tr>
<td>DEAE cellulose&lt;sup&gt;c&lt;/sup&gt;</td>
<td>510</td>
<td>1402.5</td>
<td>4.73</td>
<td>2412.3</td>
<td>1.72</td>
<td>82.8</td>
</tr>
<tr>
<td>CM-cellulose&lt;sup&gt;d&lt;/sup&gt;</td>
<td>185</td>
<td>50</td>
<td>0.39</td>
<td>72.15</td>
<td>1.44</td>
<td>24.7</td>
</tr>
<tr>
<td>Affi-Gel Blue&lt;sup&gt;e&lt;/sup&gt;</td>
<td>86</td>
<td>34.4</td>
<td>0.73</td>
<td>62.78</td>
<td>18.25</td>
<td>21.6</td>
</tr>
<tr>
<td>GSH-agarose&lt;sup&gt;f&lt;/sup&gt;</td>
<td>22</td>
<td>1.34</td>
<td>1.146</td>
<td>25.21</td>
<td>18.27</td>
<td>8.7</td>
</tr>
</tbody>
</table>

- a: Protein concentration was determined by the method of Bradford (1976).
- b: The enzyme activity was measured at 37°C and pH7.5 using 0.2 mM CDNB and 0.2 mM GSH as substrates. U = µM conjugate/minute.
- c: The chromatography was carried out at pH8.0.
- d: The chromatography was performed at pH6.7 and a KCl linear gradient was applied.
- e: The chromatography was carried out at pH8.0 and 2-20 mM GSH steps were applied.
- f: The affinity chromatography was performed at pH7.0 and the active eluant was collected by application of 5 mM GSH (pH9.6).
Table IV-2. Purification of GST-2*2 from human liver

<table>
<thead>
<tr>
<th></th>
<th>Total volume (ml)</th>
<th>Total proteina (mg)</th>
<th>Activityb (U/ml)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>770</td>
<td>20790</td>
<td>7.90</td>
<td>6083</td>
<td>0.293</td>
<td>100</td>
</tr>
<tr>
<td>DEAE cellulosec</td>
<td>2350</td>
<td>8812.5</td>
<td>1.04</td>
<td>2444</td>
<td>0.277</td>
<td>40.2</td>
</tr>
<tr>
<td>DEAE cellulosed</td>
<td>910</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CM-Sephadexe</td>
<td>900</td>
<td>585.0</td>
<td>1.24</td>
<td>1116</td>
<td>1.91</td>
<td>18.3</td>
</tr>
<tr>
<td>Affi-Gel Bluef</td>
<td>1380</td>
<td>-</td>
<td>0.355</td>
<td>479.3</td>
<td>-</td>
<td>7.9</td>
</tr>
<tr>
<td>GSH-agaroseg</td>
<td>235</td>
<td>38.8</td>
<td>1.51</td>
<td>354.9</td>
<td>9.14</td>
<td>5.8</td>
</tr>
</tbody>
</table>

a: Protein concentration was determined by the method of Bradford (1976).
b: The enzyme activity was measured at 37°C and pH7.5 using 0.2 mM CDNB and 0.2 mM GSH as substrates. U = µM conjugate/minute.
c: The chromatography was carried out at pH6.7.
d: The chromatography was performed at pH7.5.
e: The chromatography was carried out at pH8.0.
f: The chromatography was performed at pH8.0 and 2 mM GSH was applied.
g: The affinity chromatography was performed at pH7.0 and the active eluant was collected by application of 5 mM GSH (pH9.6).
Figure IV-1. Elution pattern of GST-2*1 and protein from CM cellulose (pH6.7).

Figure IV-2. Elution pattern of GST-2*1 and protein from Affi-Gel Blue (pH8.0).

Figure IV-3. Elution pattern of GST-2*1 and protein from GSH-agarose (pH7.0).
KCl gradient (0 → 0.15M)

40mM GSH

30mM GSH

20mM GSH

10mM GSH

2mM – 8mM GSH

5mM GSH
Figure IV-4. Elution pattern of GST-2*2 and protein from DEAE cellulose (pH 6.7).

Figure IV-5. Elution pattern of GST-2*2 and protein from DEAE cellulose (pH 7.5).
Figure IV-6. Elution pattern of GST-2*2 and protein from CM-Sephadex (pH8.0).

Figure IV-7. Elution pattern of GST-2*2 and protein from Affi-Gel Blue (pH8.0).

Figure IV-8. Elution pattern of GST-2*2 and protein from GSH-agarose (pH7.0).
Figure IV-9. Starch gel electrophoresis of purified GST-2*1 and GST-2*2 isozymes.
Lane 1: GST-2 from a crude liver extract
Lane 2: Purified GST-2*2
Lane 3: Purified GST-2*1 - This lane contains a small amount of purified GST-2*2 spilt over from Lane 2.

Figure IV-10. SDS/polyacrylamide gel electrophoresis of purified GST-2*1 and GST-2*2.
Lanes 1 and 5: molecular weight markers.
Lanes 2 and 4: purified GST-2*1.
Lane 3: purified GST-2*2.
Chapter 4. GST-2

Subunit molecular weight: As determined by SDS/poly-acrylamide gel electrophoresis, both GST-2*1 and GST-2*2 isozymes have an identical subunit molecular weight which was calculated to be 26,900 (Figure IV-10).

Kinetic analysis, specific activity and inhibition studies: The $K_m$ values obtained for GSH and CDNB with the GST-2*1 and GST-2*2 isozymes are shown in Table IV-3.

Table IV-3. The $K_m$ values (mM) for CDNB and GSH of the GST-2*1 and GST-2*2 isozymes.

<table>
<thead>
<tr>
<th></th>
<th>GST-2*1</th>
<th>GST-2*2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNB</td>
<td>0.067</td>
<td>0.071</td>
</tr>
<tr>
<td>GSH</td>
<td>0.153</td>
<td>0.144</td>
</tr>
</tbody>
</table>

The $K_m$ values obtained for CDNB with both GST-2*1 and GST-2*2 were much lower than those of the cationic glutathione S-transferases previously reported by other researchers. The $K_m$ values of both GST-2 isozymes were also lower than those of GST-1 reported in Chapter 3.

Unlike the GST-1 isozymes, both GST-2 isozymes were found to have glutathione peroxidase activity with cumene and t-butyl hydroperoxides as substrates (Table IV-4).
Chapter 4. GST-2

Table IV-4. Glutathione peroxidase activity of the GST-2*1 and GST-2*2 isozymes.

<table>
<thead>
<tr>
<th></th>
<th>GST-2*1a</th>
<th>GST-2*2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumen hydroperoxide</td>
<td>3.43</td>
<td>5.02</td>
</tr>
<tr>
<td>t-Butyl hydroperoxide</td>
<td>0.254</td>
<td>0.127</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a: µM/mg of protein/min.

The specific activity with several additional substrates are shown in Table IV-5.

Table IV-5. Specific activity of the GST-2*1 and GST-2*2 isozymes with different substrates.

<table>
<thead>
<tr>
<th></th>
<th>GST-2*1a</th>
<th>GST-2*2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-chloro-2,4-dinitrobenzene</td>
<td>614.0</td>
<td>322.0</td>
</tr>
<tr>
<td>1,2-dichloro-4-nitrobenzene</td>
<td>6.97</td>
<td>10.60</td>
</tr>
<tr>
<td>1,2-epoxy-3-(p-nitrophenoxy)propane</td>
<td>59.24</td>
<td>26.33</td>
</tr>
<tr>
<td>Ethacrynic acid</td>
<td>23.48</td>
<td>59.80</td>
</tr>
<tr>
<td>trans-4-phenyl-3-buten-2-one</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bromosulfophthalein</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a: µM/mg of protein/min.

GST-2 isozymes were found to have the capacity to utilize 1,2-epoxy-3-(p-nitrophenoxy)propane and ethacrynic acid which were not good substrates for the GST-1 isozymes.
The inhibitory effects of five chemicals on GST-2*1 and GST-2*2 are illustrated in Figure IV-11. Although there were some differences in the inhibition profiles of GST-2*1 and GST-2*2, the general tendency of these features was similar. In contrast, the effects of cholate, chenodeoxycholate and ellagic acid on the GST-2 isozyme were much less than those on the GST-1 isozymes.

**Heat stability:** the relative stability at 45°C and the effect of different temperatures on the GST-2*1 and GST-2*2 isozymes are illustrated in Figures IV-12A & B. The heat stability of both GST-2*1 and GST-2*2 isozymes did not differ in the present studies. Similarly the heat stability of the GST-2 isozymes did not differ significantly from those of the GST-1 isozymes.

**pH optimum:** the variations in the isozyme activities of GST-2*1 and GST-2*2 with pH are shown in Figures IV-13. The pH curve of GST-2*2 was shifted slightly higher than that of GST-2*1.

**Immunological studies:** Figure IV-14A shows double immunodiffusion of GST-2*1 and GST-2*2 against antiserum raised against GST-2*2. A clear reaction of identity between GST-2*1 and GST-2*2 was obtained. Figure IV-14C shows an electroblot of an SDS/polyacrylamide gel on which purified GST-2*1 and purified GST-1*2 had been separated. Immunological blotting with anti GST-2*2 detected only GST-2 and did not cross-react with GST-1. A similar result is shown in Figure IV-14B where anti GST-2*2 did not cross-react with GST-1*1 in an immunodiffusion experiment.
Figure IV-11. The inhibitory effects of some chemicals on GST-2*1 and GST-2*2 activity.

A: cholate
B: chenodeoxycholate
C: lithocholate 3-sulfate
D: ellagic acid
E: sulfobromophthalein

GST-2*1 (-----)
GST-2*2 (-----)
Cholate (μM)

Lithocholate 3-sulfate (μM)

Chenodeoxycholate (μM)

Ellagic acid (μM)

Bromosulfophthalein (μM)
Figure IV-12. The effect of temperature on GST-2*1 and GST-2*2.

A. incubated for 10 minutes at various temperatures.

B. incubated for various times at 45°C.

![Graph showing the effect of temperature on GST-2*1 and GST-2*2 activity.]

Figure IV-13. The effect of pH on GST-2*1 and GST-2*2 activity.

![Graph showing the effect of pH on GST-2*1 and GST-2*2 activity.]

GST-2*1 (○--○)

GST-2*2 (○--○)
A

% of activity at 4 °C

Temperature (°C)

B

% of activity at 0 min.

Time (minute)

% of maximum

pH
Figure IV-14.

A: Double immunodiffusion of anti GST-2*2 against GST-2*1 and GST-2*2.

B: Double immunodiffusion of anti GST-2*2 against GST-1*1 and GST-2*2.

C: Immunotransblot of GST-2*2 and GST-1*2 probed with anti GST-2*2.
   Lane 1: GST-1*2
   Lane 2: GST-2*2
DISCUSSION

The present experiments show that the two GST-2 isozymes studied have largely similar biochemical characteristics. In contrast, many properties of these isozymes, in particular, the subunit molecular weight, $K_m$ values for GSH and CDNB, glutathione peroxidase activity and substrate affinity were clearly different from those of the GST-1 isozymes studied in Chapter 3. Furthermore the present immunological studies show that the GST-2 isozymes are immunologically distinct from the GST-1 isozymes although both GST-2*1 and GST-2*2 do not differ from each other. These data therefore clearly support the proposal of Board (1981a) that the isozymes termed GST-1 and GST-2 are the products of different loci.

Several groups have purified and characterized the cationic glutathione S-transferases. Some of the available data that can be readily compared are shown in Table IV-5. Although there are some differences in the observed values in specific reports, the overall picture suggests that all these reports describe the properties of the isozymes termed GST-2 by Board (1981a). For example, Awasthi et al. (1980) reported that the subunit molecular weight of the cationic glutathione S-transferases, which they studied, was 24,500. In contrast, the value obtained in the present studies was 26,900. However, Awasthi et al. (1980) reported the presence of glutathione peroxidase activity which is
Table IV-5. Comparison of purified cationic glutathione S-transferase isozymes

<table>
<thead>
<tr>
<th>Research groups</th>
<th>Subunit M.W.</th>
<th>pI</th>
<th>Km (GSH) &amp; (CDNB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kamisaka <em>et al.</em> (1975)</td>
<td>25,000</td>
<td>7.8-8.8</td>
<td>- 0.40-0.63</td>
</tr>
<tr>
<td>Awasthi <em>et al.</em> (1980)a</td>
<td>24,500</td>
<td>8.5-9.5</td>
<td>- -</td>
</tr>
<tr>
<td>Pattinson (1981)</td>
<td>-</td>
<td>-</td>
<td>- 1.0</td>
</tr>
<tr>
<td>Koskelo &amp; Icén (1984)</td>
<td>(46,000-48,000)b</td>
<td>9.1</td>
<td>0.18 0.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.9</td>
<td>0.13 0.60</td>
</tr>
<tr>
<td>Stockman <em>et al.</em> (1985)</td>
<td>(B1B1)</td>
<td>8.9</td>
<td>0.4 1.0</td>
</tr>
<tr>
<td></td>
<td>(B2B2)</td>
<td>8.4</td>
<td>0.33 0.41</td>
</tr>
<tr>
<td></td>
<td>(B1B2)</td>
<td>8.75</td>
<td>0.35 0.76</td>
</tr>
<tr>
<td>GST-2a &amp; c</td>
<td>26,900</td>
<td>&gt;8.6 (2*1)</td>
<td>0.153 0.067</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2*2)</td>
<td>0.144 0.071</td>
</tr>
</tbody>
</table>

*a: glutathione peroxidase activity of the isozyme(s) was reported.*

*b: native molecular weight*

*c: the data derived from the present experiments*

Clearly a distinctive feature of the GST-2 isozymes studied here. The differences in molecular weight presumably reflect differences in laboratory methodology and standardization.

The previous reports of Kamisaka *et al.* (1975), Strange *et al.* (1984b) and Laisney *et al.* (1984) have supported the view that the cationic glutathione S-transferases are the post-translationally modified products of a single gene. In contrast, Board (1981a) suggested that the different isozymes were the homo- and hetero-dimeric combination of two allelic subunits. Board's suggestion was based on the
frequency of each proposed allele and the apparent existence of a Hardy-Weinberg equilibrium. This argument has been considerably weakened by the observations of apparently different GST-2 phenotypes in different tissues of the same individual (Chapter 2; Strange et al., 1984b; Laisney et al., 1984).

Recently Stockman et al. (1985) presented evidence that as suggested by Board (1981a) the cationic glutathione S-transferases were the product of the hetero- and homodimeric combination of two subunits. However, Stockman et al. (1985) also demonstrated that these subunits were immunologically distinct and that observation suggests that the two different subunits are the products of separate genes. The variation in expression of the GST-2 isozymes observed between different tissues of a single individual could be more readily explained if the two subunits were the product of different gene loci. However the products of these genes must be extremely similar since their molecular weights are apparently identical and in most reports the biochemical characteristics of the cationic or GST-2 isozymes are similar.

The biochemical analysis of the cationic GST-2 isozymes reported here and by others (Table IV-5) can not clearly differentiate between the one and two gene hypotheses. The slight differences observed between GST-2*1 and GST-2*2 in pH optimum and susceptibility to inhibition could clearly result from minor structural differences between different allele or gene products. The two gene
hypothesis of Stockman et al. (1985) is dependent largely on the observation of immunological dissimilarity. Since Kamisaka et al. (1975) have previously reported the immunological similarity of the cationic glutathione S-transferases, it is obvious that these questions are still unresolved. Finally the present immunological studies clearly show that two isozymes, GST-2*1 and GST-2*2 have immunological similarity. It therefore seems more likely that these isozymes are the post synthetically modified products of a single gene locus.

As described in Chapter 2, Strange et al. (1984a) reported a liver specimen without GST-2 activity but with GST-1 and GST-3 activity (Figure IV-15A). Dr. Richard Strange kindly provided an extract from that liver sample for further immunological analysis. An immunoblot of that sample (L13) and a normal control liver extract fractionated on SDS/polyacrylamide gel failed to detect any GST-2 protein in the L13 sample (Figure IV-15B). Since the GST-1 and GST-3 activities of the L13 sample were apparently quite normal, it seems unlikely that the GST-2 in this sample has been degraded. However, because this liver sample was obtained during postmortem investigations of this case and cannot be repeated, any conclusions are bound to be speculative. If L13 is an example of GST-2 deficiency, it is significant that all the cationic isozymes are affected. This finding clearly supports the suggestion that the cationic isozymes are the products of a single gene.
Figure IV-15.

A: Starch gel electrophoresis of liver samples (from Strange et al, 1984a).

- Lanes 1 and 2: Normal liver samples
- Lane 3: Liver sample L-13 showing GST-2 deficiency and normal GST-1*2-1 phenotype

B: Immunotransblot of L13 and normal liver extract fractionated by SDS/polyacrylamide gel electrophoresis, probed with anti GST-2

- Lane 1: Liver sample L13
- Lane 2: Normal liver sample
Chapter 5

The purification and biochemical characterization of the GST-3 isozyme from human lung
INTRODUCTION

Board (1981a) originally reported a glutathione S-transferase isozyme from liver and erythrocyte extracts which migrated relatively rapidly towards the anode during electrophoresis at pH8.6. Although there were slight differences in electrophoretic mobility between the two different tissues, it was suggested that this isozyme was probably the product of a single locus termed GST-3. Unlike the GST-1 and GST-2 loci, this locus did not appear to exhibit any electrophoretic polymorphism (Board, 1981a; see Chapter 1). The absence of electrophoretic polymorphism at this locus has been confirmed independently by Scott and Wright (1980) and Strange et al. (1984b). Interestingly, Strange et al. (1983) reported the presence of two electrophoretically distinct forms of the glutathione S-transferase in human erythrocytes and suggested that these may result from post-translational modification.

Additional studies reported here in Chapter 2 and the published reports of Strange et al. (1984b) and Laisney et al. (1984) have confirmed the presence of similar GST-3 isozymes in a number of tissues. In some tissues including lymphocytes, erythrocytes and lung, GST-3 appears to be the major, if not the only form, of glutathione S-transferase.

Because the lung is the initial site of exposure and uptake of a wide range of environmental xenobiotics, it is likely that the glutathione S-transferase in the lung plays
an important role in the metabolism of these compounds, for example, protection against inhaled chemical carcinogens.

As mentioned before in Chapter 1, a glutathione S-transferase may catalyze the formation of leukotriene $C_4$ (LTC$_4$), one of the slow reacting substances of anaphylaxis (SRS-A) (Samuelsson, 1983). Mannervik et al. (1984) determined if any of the six major basic cytosolic glutathione S-transferases from rat liver catalyzed the conversion of leukotriene $A_4$ methyl ester to the corresponding leukotriene $C_4$ monomethyl ester in vitro. They found that rat glutathione S-transferase '4-4', which is the isozyme with the lowest $pI$ in normal rat liver cytosol, had the greater potential activity among these isozymes. The release mechanism of SRS-A from cells has not been identified. However SRS-A has been shown to exist in leukocytes and lung in man (Samuelsson, 1983). Since the product of the GST-3 locus is expressed predominantly in lung and leukocytes, GST-3 is the most likely isozyme to be associated with LTC$_4$ synthesis in human tissues.

Marcus et al. (1978) purified and characterized glutathione S-transferase from human erythrocytes and demonstrated that the enzyme had a $pI$ of 4.5 and differed substantially from the reported characteristics of other cationic glutathione S-transferases purified from the liver which had $pI > 8$. Since the report of Marcus et al. (1978), several other anionic glutathione S-transferases have been purified from a number of tissues by several groups.
Because of the apparent similarities between the anionic glutathione S-transferase isozymes characterized from different tissues, it is likely that they are all products of the same locus, and it is highly likely that they are all identical to the isozyme previously identified as GST-3 by electrophoresis. In order to resolve this question and because of the potential importance of glutathione S-transferase in the lung, the electrophoretically fast anodal GST-3 isozyme found in human lung has been purified and its characteristics compared with the products of the GST-1 and GST-2 loci reported in Chapter 3 and Chapter 4. A comparison has also been made with some previously published data obtained for acidic glutathione S-transferases purified from other human tissues.

**MATERIALS AND METHODS**

Materials, and the procedures for the determination of glutathione S-transferase activity and protein concentration, starch gel electrophoresis, SDS/polyacrylamide gel electrophoresis have been described in detail in Chapters 2 and 3.

**Purification of the GST-3 isozyme from human lung:**

Step 1. Human lung was obtained at autopsy and stored at -20°C until the experiments were carried out. Before
the purification, the tissue was examined by starch gel electrophoresis and the presence of a single acid GST-3 isozyme was confirmed. A homogenate of lung, 386 g was made in 1,600 ml of 10 mM Tris/HCl buffer, pH8.0 (Tris/HCl buffer). The homogenate was centrifuged at 10,000 x g for 2 hours and the supernatant was filtered through a plug of cotton, and 1,500 ml of the supernatant was finally obtained. The supernatant was concentrated by precipitation with ammonium sulfate at 40% saturation. The precipitate was collected by centrifugation at 10,000 x g for 30 minutes. The precipitate was dissolved in an adequate volume of 20 mM phosphate buffer (pH6.5) and dialyzed against five changes of 5 litres of 20 mM phosphate buffer (pH6.5) for more than 24 hours.

Step 2. CM-Sephadex (Pharmacia) (approximately 600 g wet weight), equilibrated with 20 mM phosphate buffer (pH6.5), was added to the dialyzed sample and the mixture was left for 1 hour. The mixture was then filtered on a buchner funnel and the CM-Sephadex gel was washed with 20 mM phosphate buffer (pH6.5) until the eluant was free of glutathione S-transferase activity. The procedure described above was repeated three times consecutively using recycled CM-Sephadex and the eluant from each step. Finally, 2 litres of the pooled eluant were collected. Ammonium sulfate was added to the eluant to give 40% saturation and stirred at 4°C overnight. The mixture was centrifuged at 10,000 x g for 30 minutes, and the precipitate was collected and dialyzed against 20mM phosphate buffer(pH6.0).
Step 3. The dialyzed sample (270 ml) was divided equally and applied to two columns of DEAE cellulose (Whatman DE52) (3.5 x 30 cm) equilibrated with 20 mM phosphate buffer (pH6.0). The columns were extensively washed with 20 mM phosphate buffer (pH6.0) until the enzyme activity and protein (O.D.1280) were no longer eluted. The active fractions eluted from each column with 20 mM phosphate buffer were pooled to give a final volume of 380 ml. To determine if any residual enzyme remained bound to DEAE cellulose, the columns were eluted with 0.5 M KCl.

Step 4. The pooled sample was concentrated by ultrafiltration through an Amicon DIAFLO PM10 membrane, and subsequently dialyzed against five changes of 5 litres of 20 mM phosphate buffer (pH7.0). The sample (30 ml) was applied to a column of Affi-Gel Blue (BIORAD) (pH7.0) (2.3 cm x 12 cm) and 2 mM GSH (pH7.0) was used to elute the enzyme. The active fractions were pooled and again concentrated by ultrafiltration. The concentrated enzyme preparation was dialyzed against two changes of 5 litres of 20 mM phosphate buffer (pH7.0).

Step 5. The dialyzed sample (30 ml) was applied to a column of GSH-agarose (Sigma) (pH7.0) (2.3 cm x 6 cm), and was washed with 20 mM phosphate buffer (pH7.0). GST-3 isozyme was eluted from the GSH-agarose column by the addition of 5 mM GSH in 50 mM Tris (pH9.6).

Substrate specificity, heat stability, pH optimum, kinetic constants and susceptibility to inhibition were
evaluated by methods previously described in detail in Chapter 3. In addition to the inhibitors studied in previous chapters, indomethacin was also used at concentrations ranging up to 120 µM.

Immunological studies were carried out by the methods described in Chapters 3 and 4.

RESULTS

Purification of the GST-3 isozyme from human lung:
The summary of the purification steps are shown in Table V-1. Almost all haemoglobin and components more cationic than the GST-3 isozyme were removed from the sample by CM-Sephadex (pH6.0) at the second step. Components more anionic than the GST-3 isozyme were eliminated by chromatography on DEAE cellulose (pH6.0) (Figure V-1). The elution pattern from Affi-Gel Blue and GSH-agarose are shown in Figures V-2 and V-3 respectively. The GST-3 isozyme prepared by this procedure was found to be free of contaminants when examined by SDS/polyacrylamide gel electrophoresis (Figure V-4).

Subunit molecular weight: A single subunit was identified by SDS/polyacrylamide gel electrophoresis after reduction of disulphide bonds at 100°C. The subunit molecular weight was calculated to be 25,100 (Figure V-4).
Table V-1. Purification of GST-3 from human lung

<table>
<thead>
<tr>
<th></th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Activity (U/ml)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1500</td>
<td>35130</td>
<td>1.015</td>
<td>15225</td>
<td>0.43</td>
<td>100</td>
</tr>
<tr>
<td>CM-Sephadex</td>
<td>2000</td>
<td>5240</td>
<td>0.500</td>
<td>10000</td>
<td>1.91</td>
<td>65.7</td>
</tr>
<tr>
<td>DEAE cellulose</td>
<td>380</td>
<td>1033.6</td>
<td>17.85</td>
<td>6783</td>
<td>6.56</td>
<td>44.6</td>
</tr>
<tr>
<td>Affi-Gel Blue</td>
<td>310</td>
<td>47.12</td>
<td>7.88</td>
<td>2442.8</td>
<td>51.84</td>
<td>16.0</td>
</tr>
<tr>
<td>GSH-Agarose</td>
<td>136</td>
<td>19.43</td>
<td>9.51</td>
<td>1293.4</td>
<td>66.60</td>
<td>8.5</td>
</tr>
</tbody>
</table>

a: Protein concentration was determined by the method of Bradford (1976).
b: The enzyme activity was measured at 37°C and pH7.5 using 0.2 mM CDNB and 0.2 mM GSH as substrates. \( U = \mu M \) conjugate/minute.
c: This step was performed at pH6.5.
d: The chromatography was carried out at pH6.0.
e: Affi-Gel Blue (BIORAD) was used at pH7.0 and the enzyme was eluted by the addition of 2 mM GSH (pH7.0).
f: The affinity chromatography was performed at pH7.0 and the enzyme was eluted by the application of 50 mM Tris containing 5 mM GSH (pH9.6).
Figure V-1. Elution pattern of GST-3 and protein from DEAE cellulose (pH6.0).

Figure V-2. Elution pattern of GST-3 and protein from Affi-Gel Blue (pH7.0).

Figure V-3. Elution pattern of GST-3 and protein from GSH-agarose (pH7.0).
Figure V-4. SDS/polyacrylamide gel electrophoresis of purified GST-3.

Lanes 1 & 5: Molecular weight markers
Lane 2  : GST-1
Lane 3  : GST-2
Lane 4  : GST-3
Kinetic analysis, specific activity and inhibition studies: The $K_m$ value of GST-3 for GSH was 0.27 mM and that for CDNB was 0.17 mM. Glutathione peroxidase activity could not be detected with tert-butyl hydroperoxide, cumene hydroperoxide or $H_2O_2$ as substrates. The specific activities for several additional substrates are shown in Table V-2.

Table V-2. Specific activities of GST-3 with different substrates

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Specific activity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-chloro-2,4-dinitrobenzene</td>
<td>49.89</td>
</tr>
<tr>
<td>1,2-dichloro-4-nitrobenzene</td>
<td>2.37</td>
</tr>
<tr>
<td>1,2-epoxy-3-(p-nitrophenoxy)propane</td>
<td>0</td>
</tr>
<tr>
<td>bromosulfophthalein</td>
<td>0</td>
</tr>
<tr>
<td>trans-4-phenyl-3-buten-2-one</td>
<td>0</td>
</tr>
<tr>
<td>ethacrynic acid</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$: specific activity is expressed as µmoles/min./mg of protein, and the detailed assay procedures are described in Chapter 3.

The inhibitory effects of five chemicals on GST-3 are shown in Table V-3 where the concentrations of the inhibitors producing 50% inhibition of enzyme activity ($I_{50}$ value, µM) are compared with the data for GST-1 and GST-2 which were calculated from Chapters 3 and 4.
Table V-3. The inhibition of GST-3 (I<sub>50</sub> value)<sup>a</sup>

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>GST-1&lt;sup&gt;b&lt;/sup&gt;</th>
<th>GST-2&lt;sup&gt;c&lt;/sup&gt;</th>
<th>GST-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholate</td>
<td>95</td>
<td>N.E.&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Chenodeoxycholate</td>
<td>50</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Lithocholate 3-sulfate</td>
<td>20</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>85</td>
<td>&gt;200</td>
<td>105</td>
</tr>
<tr>
<td>Bromosulfophthalein</td>
<td>5</td>
<td>10</td>
<td>50</td>
</tr>
</tbody>
</table>

<sup>a</sup> the concentration of inhibitor producing 50% inhibition of enzyme activity.
<sup>b</sup> the data was calculated from the results of GST-1*1 in Chapter 3.
<sup>c</sup> the data was calculated from the results of GST-2*1 in Chapter 4.
<sup>d</sup> less than 10% inhibition was obtained with a final solution concentration of 200 mM.

Because of its role as an anti-inflammatory drug and because of the possibility that GST-3 is involved in leukotriene C<sub>4</sub> synthesis, indomethacin was examined to determine if it was a potent inhibitor of the human glutathione S-transferases. The results illustrated in Figure V-5 clearly show that indomethacin does not substantially inhibit the activity of either GST-1, GST-2 or GST-3.

Heat stability: the relative stability of GST-3 at 45°C and the effect of different temperatures on GST-3 are shown respectively in Figures V-6A & V-6B. The GST-3 isozyme appears to have a heat stability profile similar to those of GST-1 and GST-2 (Chapters 3 and 4).
Figure V-5. The effect of indomethacin on the activity of GST isozymes.
The graph illustrates the effect of indomethacin concentration on GST activity (% of control) for three different GST forms: GST-1, GST-2, and GST-3. The x-axis represents the concentration of indomethacin in µM, ranging from 0 to 120, while the y-axis represents GST activity, decreasing from 100% at the 0 µM to lower levels as the concentration increases. The data points for GST-1, GST-2, and GST-3 show a downward trend as the indomethacin concentration increases, indicating a suppression of GST activity.
Figure V-6. The effect of temperature on GST-3 activity.
A. incubated for 10 minutes at various temperatures.
B. incubated for various times at 45°C.

Figure V-7. The effect of pH on GST-3 activity.
Figure V-8.

A) Immunodiffusion of purified GST-1*2, GST-2*2 and GST-3 against antibody raised against GST-3.

B) Immunotransblot of GST-1*1, GST-2*1 and GST-3 with anti GST-3.

Lane 1: GST-1*1
Lane 2: GST-2*1
Lane 3: GST-3
Chapter 5. GST-3

\textbf{pH optimum:} the variation of GST-3 activity with pH is shown in Figure V-7. The GST-3 isozyme appears to have maximum activity between pH 7.5 and pH 8.0.

\textbf{Immunological studies:} The immunodiffusion experiments shown in Figure V-8A show that an antibody against purified GST-3 from human lung did not cross-react with either GST-1*2 or GST-2*2. Other immunoblotting experiments also indicated that anti GST-3 did not cross-react with GST-1*1 or GST-2*1 fractionated on SDS/polyacrylamide gel (Figure V-8B).

\textbf{DISCUSSION}

The GST-3 isozyme purified from lung had a subunit molecular weight of 25,100 which clearly differed from that of the GST-1 isozymes (27,500) and GST-2 isozymes (26,900). The \( K_m \) values of the GST-3 isozyme for GSH and CDNB were found to be different from those of the GST-1 and GST-2 isozymes (Chapters 3 and 4). In these experiments, the GST-3 isozyme failed to exhibit glutathione peroxidase activity which, though similar to GST-1, is unlike GST-2 which has strong glutathione peroxidase activity with \( t \)-butyl and cumen hydroperoxides as substrates. The heat stability and pH optimum of the GST-3 isozyme were generally found to be similar to those of the GST-1 and GST-2
isozyymes. However GST-3 could be discriminated from GST-1 and GST-2 by its susceptibility to inhibition by different compounds.

Recently, Tahir et al. (1985) was able to discriminate between three types of human glutathione S-transferase by the use of some inhibitors including Cibacron blue and tributyltin acetate. Cibacron blue was a potent inhibitor of the near-neutral and acidic isozymes and tributyltin acetate inhibited the basic isozyme (Tahir et al., 1985). Pattinson (1981) showed that an anionic glutathione S-transferase from human liver cytosol was inhibited by the primary bile acid, cholate, but not by chenodeoxycholate. The present studies did not confirm the findings of Pattinson (1981) and only GST-1 (a near-neutral isozyme) was noticeably inhibited by those compounds.

The antiserum raised against GST-3 failed to cross-react with GST-1 and GST-2. Furthermore, although not shown specifically here, antisera raised against GST-1 and GST-2 failed to cross-react with GST-3. These observations, when considered in relation to the other biochemical differences, clearly confirm the original proposal of Board (1981a) that GST-3 is the product of a separate gene.

The present results can be compared with apparently similar isozymes purified from a range of tissues by other groups. Table V-4 shows the comparison of molecular weight, pI, and $K_m$ values of anionic glutathione S-transferase purified from various human tissues with GST-3.
Table V-4. Comparison of purified anionic glutathione S-transferases and GST-3

<table>
<thead>
<tr>
<th>Tissues</th>
<th>M.W.</th>
<th>pI</th>
<th>$K_m$ (GSH)</th>
<th>$K_m$ (CDNB)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>erythrocytes</td>
<td>47,500</td>
<td>4.5</td>
<td>-</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>liver</td>
<td>46,000</td>
<td>4.6</td>
<td>0.18</td>
<td>0.83</td>
<td>2</td>
</tr>
<tr>
<td>placenta</td>
<td>(23,400)</td>
<td>4.8</td>
<td>0.5</td>
<td>2.1</td>
<td>3</td>
</tr>
<tr>
<td>liver</td>
<td>(23,000)</td>
<td>4.60</td>
<td>-</td>
<td>0.4</td>
<td>4</td>
</tr>
<tr>
<td>lung</td>
<td>46,000</td>
<td>4.62</td>
<td>0.13</td>
<td>1.6</td>
<td>5</td>
</tr>
<tr>
<td>placenta</td>
<td>45,000</td>
<td>4.65</td>
<td>0.11</td>
<td>0.91</td>
<td>6</td>
</tr>
<tr>
<td>lens</td>
<td>46,000</td>
<td>4.7</td>
<td>0.3</td>
<td>0.4</td>
<td>7</td>
</tr>
<tr>
<td>kidney</td>
<td>46,000</td>
<td>4.9</td>
<td>0.057</td>
<td>1.1</td>
<td>8</td>
</tr>
<tr>
<td>lung</td>
<td>46,000</td>
<td>4.9</td>
<td>0.078</td>
<td>0.98</td>
<td>8</td>
</tr>
<tr>
<td>spleen</td>
<td>46,000</td>
<td>4.9</td>
<td>0.083</td>
<td>1.0</td>
<td>8</td>
</tr>
<tr>
<td>placenta</td>
<td>46,000</td>
<td>4.9</td>
<td>0.097</td>
<td>1.1</td>
<td>8</td>
</tr>
<tr>
<td>lung</td>
<td>(22,000)</td>
<td>4.9</td>
<td>0.32</td>
<td>0.68</td>
<td>9</td>
</tr>
<tr>
<td>fetal fibroblasts</td>
<td>-</td>
<td>4.5</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>cornea</td>
<td>46,000</td>
<td>4.4</td>
<td>0.57</td>
<td>0.95</td>
<td>11</td>
</tr>
<tr>
<td>lung c</td>
<td>(25,100)</td>
<td>-</td>
<td>0.27</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>

a: value in parenthesis is subunit molecular weight
c: present study
There are clearly some differences between the results of different laboratories. However there are also some notable similarities between these observations. Since the acidic isozyme reported is the predominant form found in all the tissues listed except liver, it seems likely that all these reports are describing the same isozyme. The immunological similarities of erythrocyte and placental glutathione S-transferase were reported by Guthenberg and Mannervik (1981) and also those between lung and placental glutathione S-transferases were described by Dao et al. (1984). These reports and the comparison of published data presented here in Table V-4 clearly suggest that the acidic glutathione S-transferase isozyme found predominantly in erythrocytes, lung, placenta and lymphocytes is the product of a single locus that was originally termed GST-3 by Board (1981a). The survey of tissues carried out in Chapter 2 showed that the GST-3 isozyme was expressed in every tissue examined.

Strange et al. (1983) reported two different forms of erythrocyte GST-3 by using starch gel electrophoresis modified from the method of Board (1980). They concluded that the two enzyme forms in erythrocytes are due to post-synthetic modification because this phenomenon could not be explained genetically and many post-translational modifications result in increased anodal mobility. In the present experiments, only one band for GST-3 was detected after electrophoresis. As reported in Chapter 2, a very fast
band which migrates toward the anode on starch gels was found in relatively fresh samples including lung but not in erythrocytes. This isozyme was called GST-6 and is described in more detail in Chapter 8. In the present experiments GST-6 was only a minor component and was separated from lung GST-3 during purification.

Wu and Mathews (1983) reported that indomethacin, a non-steroidal anti-inflammatory drug which is known to inhibit prostaglandin synthesis, also inhibits glutathione S-transferases from rat liver. Subsequently, Nicolls and Ahokas (1984) have partially confirmed that finding and have shown that although most glutathione S-transferase isozymes in rat liver are unaffected at low concentrations of indomethacin, one particular isozyme was significantly inhibited by concentrations as low as 0.2 µM. Since GST-3 is the major glutathione S-transferase isozyme expressed in human leukocytes and lungs, it is conceivable that the enzyme is responsible for the formation of LTC4. Thus the primary aim of the present experiment was to examine if GST-3 was susceptible to inhibition by indomethacin. The results indicate that GST-3 and also GST-1 and GST-2 are unaffected by indomethacin, and therefore it is unlikely that indomethacin has a direct effect on this aspect of leukotriene metabolism in man.

Because of the ease in obtaining blood samples, a number of groups have investigated the effects of various physiological, clinical and environmental factors on the level of GST-3 activity in erythrocytes. Two groups have
studied the effect of age and sex on the glutathione S-transferase activity in erythrocytes. Strange et al. (1980) found that the catalytic properties of the enzyme from fetal erythrocytes were similar to those of the enzyme from adult erythrocytes. In contrast, Carmagnol et al. (1981) reported that erythrocyte glutathione S-transferase activity decreased significantly during the first weeks of life and remained constant thereafter. Carmagnol et al. (1981) also reported similar levels of activity in males and females. However, Strange et al. (1982) reported significantly higher levels of erythrocyte glutathione S-transferase activity in females than in males. Increased activities of GST-3 were reported in blood cells and/or erythrocytes from uraemic patients (El-Rashidy et al., 1984), from newborns with hyperbilirubinaemia and from haemodialyzed patients suffering renal dysfunction (Carmagnol et al., 1981). In addition, patients with Fanconi's anaemia have been reported to have elevated levels of GST-3 activity in erythrocytes (Dallapiccola et al., 1984).

Scott and Wright (1980) reported that the level of erythrocyte glutathione S-transferase activity varied more than six-fold in different individuals, although the enzyme was homogeneous on starch gel electrophoresis. Furthermore, they suggested that the activity levels of this enzyme were inherited, since, in family studies, the levels of husband and wife were not correlated (r = -0.054, 56df) but those of children were correlated with the mean levels of their
parents \((r = 0.511, 41\text{df})\). Wide variation in the activity of platelet glutathione S-transferase, similar to the variation occurring in erythrocytes has been reported by Rogerson et al. (1984).

A similar variation in glutathione S-transferase activity towards trans-stilbene oxide in mononuclear leukocytes was observed by Seidegard and Pero (1985). Just as Scott and Wright showed the hereditary transmission of glutathione S-transferase activity levels in erythrocytes, Seidegard and Pero (1985) clearly demonstrated the hereditary characteristics of glutathione S-transferase activity in leukocytes. In their experiments, the subjects could be divided into three groups - very high, high, and low activity. It was suggested that the levels of glutathione S-transferase activity were controlled by a single locus with alleles for high and low activity. This suggestion was well supported by family studies and by the distribution of these phenotypes in the population in accordance with a Hardy-Weinberg equilibrium. Although all the available evidence suggests that GST-3 is the product of a single gene, it is evident that there may be allelic variants that have large differences in their activity.

Some groups have investigated the levels of GST-3 activity in certain environmental circumstances. Kilpikari and Savolainen (1984) described decreased activities of erythrocyte GST-3 in workers exposed to hot rubber fumes. However, Manchester and Jackoby (1982) did not find significantly different glutathione S-transferase activities in
placentas from smoking and non-smoking women. It is evident that considerable care needs to be taken when reported changes in GST-3 levels are interpreted. As discussed earlier, GST-3 levels may be under genetic control and the inadvertent selection of 'high' or 'low' individuals in different experimental groups could significantly bias the outcome.

Since erythrocytes, platelets and leukocytes all express GST-3 as their major glutathione S-transferase product, it seems likely that all these reports describe the same genetic variability. The presence of individuals that are relatively deficient in GST-3 in the population may be significant. Board (1981a & b) demonstrated that a common deficiency of GST-1 occurs and suggested that this deficiency may place affected individuals at a relatively greater risk if they are exposed to elevated levels of certain electrophilic carcinogens. A similar argument could be made for deficiencies of GST-3 and could be even more significant since GST-3 is the major, if not the only form, of glutathione S-transferase in many tissues. The relatively high risks posed by GST-1 or GST-3 deficiencies may be even higher if some individuals are deficient in both GST-1 and GST-3. In addition, if GST-3 does play a significant role in leukotriene C₄ synthesis, then the inheritance of GST-3 deficiency may affect the production of SRS-A and thus may be of considerable physiological and/or pathological significance.
The purification and biochemical characterization of GST-4, a muscle specific glutathione S-transferase isozyme
INTRODUCTION

As described in Chapter 2, three new isozymes were found and one of them was expressed in only skeletal and cardiac muscle. Laisney et al. (1984) also reported a new isozyme in several tissues which they suggested can form a heterodimer with GST-1. They termed it GST-4, and although it had a similar electrophoretic mobility, it is not yet clear if that isozyme is identical to the GST-4 identified in Chapter 2.

Ishikawa and Sies (1984) and Tu et al. (1984) have purified the glutathione S-transferases from rat heart. They found that the isozyme pattern of rat heart was different from that of liver. However, the glutathione S-transferases from human skeletal and cardiac muscle have not previously been purified and characterized.

In the present experiments, the isozyme identified as GST-4 in Chapter 2 was purified from human skeletal muscle and its characteristics compared with those of GST-1, GST-2 and GST-3 reported in the previous chapters.

MATERIALS AND METHODS

Materials, and the procedures for starch gel electrophoresis, SDS/polyacrylamide gel electrophoresis, the determination of glutathione S-transferase activity and protein concentration have been described in detail in Chapters 2 and 3.
Purification of GST-4 from human skeletal muscle:

Step 1. Human muscle was obtained during postmortem examination and stored at -20°C. The presence of GST-4 in the sample was confirmed by starch gel electrophoresis and histochemical staining (Chapter 2). A homogenate of skeletal muscle (108 g) was made in four volumes of 10 mM Tris/HCl buffer (pH 8.0) by a blender and a motor-driven teflon-glass homogenizer. The homogenate was centrifuged at 10,000 x g for 2 hours and the supernatant was filtered through a plug of cotton.

Step 2. Solid ammonium sulfate was slowly added to the supernatant to give 40% saturation. The mixture was gently stirred overnight at 4°C and then centrifuged at 10,000 x g for 30 minutes. The precipitate was dissolved in an adequate volume of 10 mM Tris/HCl buffer (pH 8.0) and dialyzed against five changes of 5 litres of 10 mM Tris/HCl buffer (pH 8.0) for 24 hours. The dialyzed sample (88 ml) was applied to a column of DEAE cellulose (3.5 cm x 30 cm) equilibrated with 10 mM Tris/HCl (pH 8.0). The column was extensively washed with the same buffer until the enzyme activity and protein were no longer detected in the eluant. GST-4 was eluted from the column with a 1,240 ml linear gradient between 0 and 0.3 M KCl in 10 mM Tris/HCl (pH 8.0). The active fractions were pooled with a final volume of 147.5 ml.

Step 3. The sample was concentrated by ultrafiltration on a DIAFLO PM10 membrane (Amicon) and dialyzed against 10
mM phosphate buffer (pH7.0). The dialyzed sample was applied to a column of Affi-Gel Blue (2.3 cm x 12 cm) (pH7.0) and the gel was washed extensively until glutathione S-transferase activity and protein were no longer detected in the eluant. GST-4 was eluted from the column with 2 mM GSH (pH7.0). The active fractions were again pooled to give a total volume of 82 ml which was concentrated by ultrafiltration to 15 ml.

Step 4. The sample was dialyzed against 10 mM sodium phosphate buffer (pH6.0) and the dialyzed sample was applied to a 2.3 cm x 12 cm column of Affi-Gel Blue equilibrated with 10 mM phosphate buffer (pH6.0). The gel was extensively washed with 10 mM phosphate buffer (pH6.0) until glutathione S-transferase activity and protein were no longer detected. At this pH, GST-4 did not bind to the column. In order to confirm that GST-4 was not retained on the column, 2 mM GSH (pH6.0) was applied to the column following by 0.05 M glycine/NaOH buffer (pH9.5). No further GST-4 was eluted under these conditions. The active eluant, which had passed straight through the column, was collected and its volume was 24 ml.

Step 5. After the sample was dialyzed against 10 mM phosphate buffer (pH7.0), it was applied to a column of DEAE cellulose (pH7.0) (3.5 cm x 30 cm). The gel was extensively washed until the enzyme activity of the glutathione S-transferase and protein were as no longer detected. The GST-4 isozyme was eluted with a linear gradient between
0 and 0.3 M KCl in 10 mM phosphate buffer (pH 7.0). The active fractions were collected and pooled to give a final volume of 64 ml.

Step 6. The sample was concentrated by ultrafiltration and dialyzed against two changes of 5 litres of 10 mM phosphate buffer (pH 7.0). The dialyzed sample was applied to a column of GSH-agarose (pH 7.0) (2.3 cm x 6 cm) and the column was washed until no further glutathione S-transferase activity or protein could be detected in the eluant. GST-4 was eluted from the column with 50 mM Tris/HCl containing 5mM GSH (pH 9.6). The purified GST-4 was eluted in a total volume of 10 ml.

The detailed procedures for evaluating substrate specificity, kinetic analysis, inhibition studies, heat stability, pH optimum and immunodiffusion using antisera raised against GSTs-1, 2 and 3 have been described in Chapter 3.

RESULTS

Purification of the GST-4 isozyme from skeletal muscle: A summary of the purification is shown in Table VI-1. The elution patterns for the purification of GST-4 isozyme are shown in Figures VI-1 to VI-4. Because the muscle used in these experiments did not express either GST-1 or GST-2,
Table VI-1. Purification of GST-4 from human skeletal muscle

<table>
<thead>
<tr>
<th></th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Activity (U/ml)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>445</td>
<td>4227.5</td>
<td>0.232</td>
<td>103.24</td>
<td>0.024</td>
<td>100</td>
</tr>
<tr>
<td>DEAE cellulose</td>
<td>147.5</td>
<td>2065.0</td>
<td>0.621</td>
<td>91.60</td>
<td>0.044</td>
<td>88.7</td>
</tr>
<tr>
<td>Affi-Gel Blue</td>
<td>82.0</td>
<td>60.68</td>
<td>0.683</td>
<td>56.01</td>
<td>0.924</td>
<td>54.3</td>
</tr>
<tr>
<td>Affi-Gel Blue</td>
<td>24.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DEAE cellulose</td>
<td>64.0</td>
<td>24.96</td>
<td>0.745</td>
<td>47.68</td>
<td>1.910</td>
<td>46.2</td>
</tr>
<tr>
<td>GSH-agarose</td>
<td>10.0</td>
<td>0.05</td>
<td>0.375</td>
<td>3.75</td>
<td>75.0</td>
<td>3.6</td>
</tr>
</tbody>
</table>

a: Protein concentration was determined by the method of Bradford (1976).
b: The enzyme activity was measured at 37°C and pH7.5 using 0.2 mM CDNB and 0.2 mM GSH as substrates. U = µM conjugate/minute.
c: The chromatography was carried out at pH8.0 and a linear KCl gradient was applied.
d: The chromatography was performed at pH7.0 and 2 mM GSH (pH7.0) was applied.
e: The chromatography was carried out at pH6.0.
f: The chromatography was performed at pH7.0 and a KCl gradient was applied.
g: The affinity chromatography was performed at pH7.0 and the enzyme was eluted by application of 5 mM GSH (pH9.6).
Figure VI-1. Elution pattern of GST-4 and protein from DEAE cellulose (pH8.0).

Figure VI-2. Elution pattern of GST-4 and protein from Affi-Gel Blue (pH7.0).
Chapter 6. GST-4

Figure VI-3. Elution pattern of GST-4 and protein from DEAE cellulose (pH7.0).

Figure VI-4. Elution pattern of GST-4 and protein from GSH-agarose (pH7.0).
KCl gradient (0 → 0.3M)

![Graph 1: GST activity vs. Fraction number](image1)

GST activity (x10^{-1} U/min/ml) vs. Fraction number

5mM GSH

![Graph 2: GST activity vs. Fraction number](image2)

GST activity (x10^{-1} U/min/ml) vs. Fraction number

O.D.280
the major components with glutathione S-transferase activity, which had to be removed from the GST-4 isozyme, were only the more anionic isozymes, GST-3 and GST-6. These isozymes were separated by Affi-Gel Blue (pH 7.0) and DEAE cellulose (pH 7.0) chromatography. The purity of the GST-4 isozyme was judged by SDS/polyacrylamide gel electrophoresis (Figure VI-5).

Subunit molecular weight: A single protein band was observed after reduction of disulfide bonds at 100°C and electrophoresis on SDS/polyacrylamide gels. The subunit molecular weight was calculated to be 27,300 which is slightly lighter than the subunit molecular weight of GST-1 (27,500) (Figure VI-5).

Kinetic analysis, specific activity and inhibition studies: The $K_m$ value of GST-4 for GSH was 0.154 mM and that for CDNB was 0.333 mM. The specific activity with some additional substrates is shown in Table VI-2.

Glutathione peroxidase activity could not be detected with cumen hydroperoxide, $t$-butyl hydroperoxide or $H_2O_2$ as substrates. In this respect GST-4 appeared to be similar to GST-1 and GST-3.

The inhibitory effects of five chemicals on the GST-4 isozyme are shown in Figure VI-6. The inhibitory profile of GST-4 was similar to that of GST-3 except for bromosulfophthalein. The $I_{50}$ value of bromosulfophthalein for GST-4 (6.5 $\mu$M) was much lower than that for GST-3 (50 $\mu$M).
Figure VI-5. SDS/polyacrylamide gel electrophoresis of purified GST-4

Lane 1: GST-4
Lane 2: GST-1
Figure VI-6. The inhibitory effect of some chemicals on GST-4 activity.

A: cholate
B: chenodeoxycholate
C: lithocholate 3-sulfate
D: ellagic acid
E: bromosulfophthalein
The variation in GST activity with various concentrations of compounds is shown in Figure 11.3. The compounds appear to have a dose-dependent effect on GST activity between 10 and 200 μM. The activity decreases significantly with increasing concentrations of the compounds.
Table VI-2. Specific activity of GST-4 with some substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>GST-4 (^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-chloro-2,4-dinitrobenzene</td>
<td>7.950</td>
</tr>
<tr>
<td>1,2-dichloro-4-nitrobenzene</td>
<td>0.249</td>
</tr>
<tr>
<td>1,2-epoxy-3-(p-nitrophenoxy)propane</td>
<td>0</td>
</tr>
<tr>
<td>ethacrynic acid</td>
<td>0</td>
</tr>
<tr>
<td>trans-4-phenyl-3-buten-2-one</td>
<td>0</td>
</tr>
<tr>
<td>bromosulfophthalein</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{a}\) μM/mg of protein/min.

**Heat stability:** The relative stability of the GST-4 isozyme at 45°C and the effect of different temperatures on GST-4 are shown in Figure VI-7A & VI-7B respectively. GST-4 appears to have a heat stability profile similar to those of GST-1, GST-2 and GST-3 (Chapters 3, 4 and 5).

**pH optimum:** The variation of GST-4 activity with pH is shown in Figure VI-8. The GST-4 isozyme appears to have a maximum activity between pH7.5 and pH8.0.

**Immunological studies:** Figure VI-9 show double immunodiffusion experiments using antisera raised against GST-1, GST-2 and GST-3. The purified GST-4 did not cross-react with any of these antisera.
Figure VI-7. The effect of temperature on GST-4.
A. incubated for various times at 45°C. 
B. incubated for 10 minutes at various temperatures.

Figure VI-8. The effect of pH on GST-4 activity.
Figure VI-9. Double immunodiffusion of anti GST-1, anti GST-2 and anti GST-3 against GST-4.

A) anti GST-1 against GSTs-1*1, 2*2, 3 and 4
B) anti GST-2 against GSTs-1*1, 2*2, 3 and 4
C) anti GST-3 against GSTs-1*1, 2*2, 3 and 4
DISCUSSION

Although glutathione S-transferase isozymes have been purified from rat heart (Ishikawa et al., 1984; Tu et al., 1984), the human muscle specific form of glutathione S-transferase identified in Chapter 2 as GST-4 and the same isozyme possibly detected by Laisney et al. (1984), has not previously been purified and characterized.

The main purpose of this experiment was to determine if GST-4 is an additional new isozyme or if GST-4 results from the post-translational modification or recombination of subunits from GSTs-1, 2 or 3. Strange et al. (1984b) also detected a new form of glutathione S-transferase in muscle by starch gel electrophoresis, and concluded that this isozyme was a secondary isozyme derived from GST-3 because the electrophoretic mobility of GST-3 varies slightly in different tissues. In the present experiments, although the biochemical characteristics of GST-4 were found to be similar to those of GST-3, the subunit molecular weight of GST-4 (27,300) is apparently greater than that of GST-3 (25,100). The subunit size of GST-4 falls between those of GST-1 (27,500) and GST-2 (26,900). Because only a single band of protein was observed after reduction with β-mercaptoethanol and SDS/polyacrylamide gel electrophoresis, it is clear that GST-4 is not a heterodimer derived from GST-1, GST-2 or GST-3 subunits. In addition, some biochemical features appear to distinguish GST-4
from other previously characterised isozymes. For example, GST-1 and GST-4 isozymes can be distinguished on the basis of the susceptibility of GST-1 to inhibition by cholate. Similarly GST-2 isozymes have glutathione peroxidase activity and this property is not shared by GST-4. The finding that antiserum against GST-1, 2 or 3 does not cross react with purified GST-4 also strongly supports the proposal that GST-4 is the product of an additional glutathione S-transferase gene that can be termed GST-4.

As noted in Chapter 2, the GST-4 isozyme was only observed in skeletal and cardiac muscle tissues. Laisney et al. (1984) also observed an electrophoretically similar isozyme in muscle tissue, which they also termed GST-4. In contrast Laisney et al. (1984) suggested that the isozyme was detected in other tissues, and could form a heterodimer with GST-1 subunits. No isozymes with the expected electrophoretic mobility of a GST-1/GST-4 heterodimer were observed in the present experiments which included the study of muscle samples from individuals who expressed GST-1. It is, therefore, not clear if the 'GST-4' reported by Laisney et al. (1984) corresponds to the GST-4 isozyme characterised here.

The significance and role of GST-4 is not yet clear. Since muscle tissue is not known to be a major site for the detoxification and metabolism of xenobiotics, and since GST-4 appears to be primarily restricted to muscle tissue, it is possible that it plays some specialized role. Studies
of rat liver glutathione S-transferases, reviewed in Chapter 1, have shown that the glutathione S-transferases may have a significant role as binding proteins and can potentially function as regulators of the intracellular transport of organic anions. Ketterer (1976) reported that rat liver glutathione S-transferase was a heme binding protein which possibly facilitated the transport of heme from the mitochondria to the cytoplasm. These concepts suggested a possible role for glutathione S-transferase in erythrocytes and Harvey and Beutler (1982) suggested that GST-3 functions physiologically as a hemin binding and/or transport protein in developing erythroid cells. It is possible that GST-4 could play a similar role in the supply of heme for myoglobin in muscle, however this possibility requires further investigation.

The results obtained in the present investigations suggest that GST-4 is only expressed in muscle tissue. If this proves to be a consistent finding, then GST-4 may warrant further investigation as a diagnostic marker in muscular and heart disease.
Chapter 7

The purification and biochemical characterization of the GST-5 isozyme from human brain
INTRODUCTION

Preliminary studies of glutathione S-transferase expression in different tissues (Chapter 2) revealed an isozyme termed GST-5 which is only expressed in brain and migrates between the two allelic products of the GST-1 locus (GST-1*1 and GST-1*2), on starch gel electrophoresis (pH 8.3).

Laisney et al. (1984) reported an isozyme they termed GST-5 in brain. However this isozyme was apparently also expressed weakly in lung. Theodore et al. (1985) purified three glutathione S-transferase isozymes from human brain. They found that human brain contained one cationic (pI 8.3) and two anionic (pI 5.5 and 4.6) isozymes. Since the isoelectric point of the GST-5 isozyme seems to be near-neutral when compared with the GST-1 isozymes on starch gel electrophoresis, it is not immediately clear which isozymes were purified by Theodore et al. (1985).

The electrophoretic mobility of GST-5 in brain tissue is clearly shown in Figure II-2A of Chapter 2. This isozyme was not detected in half of the brain samples examined. Furthermore, GST-1 isozymes were present in all but one of the samples expressing GST-5. Because of these results, it is not clear if GST-5 is related to the GST-1 isozymes in brain or if GST-5 has an independent origin. In order to clarify this issue, the present experiments were undertaken and GST-5 was purified from human brain.
MATERIALS AND METHODS

Materials, and the detailed procedures for the determination of glutathione S-transferase activity, protein concentration, starch gel electrophoresis, and SDS/polyacrylamide gel electrophoresis have been described in Chapters 2 and 3.

Purification of GST-5 from human brain:

Step 1. A homogenate of human brain (663.88 g) was made in four volumes of 10 mM Tris/HCl buffer (pH 8.0) by an electric blender and a motor-driven teflon-glass homogenizer. The homogenate was centrifuged at 10,000 x g for 2 hours and the supernatant was filtered through a plug of cotton to remove lipid material. The final volume of the extract was 2,410 ml.

Step 2. Solid ammonium sulfate was added to the extract to make 40% saturation and the mixture was gently stirred overnight. The mixture was centrifuged at 10,000 x g for 30 minutes and the precipitate was collected. The precipitate was dissolved with an adequate volume of 10 mM Tris/HCl buffer (pH 8.0) and dialyzed against five changes of 5 litres of 10 mM Tris/HCl buffer (pH 8.0). The dialyzed sample (420 ml) was divided equally and each sample was applied to columns (3.5 cm x 30 cm) of DEAE cellulose (I) equilibrated in 10 mM Tris/HCl buffer, pH 8.0. The two columns were washed extensively with Tris buffer, and a 2
l litre linear gradient between 0 and 0.1 M KCl in 10 mM Tris/HCl buffer (pH8.0) was passed through each column. After the first gradient, a second gradient between 0.1 M and 0.3 M KCl was applied to each column. The active fractions were pooled in five groups, Peak (I) fractions 24-40 (410 ml), Peak (II) fractions 140-157 (460 ml), Peak (III) fractions 161-171 (280 ml), Peak (IV) fractions 175-187 (285 ml), Peak (V) fractions 191-229 (925 ml). Each pooled sample was concentrated by ammonium sulfate precipitation at 40% saturation. The precipitates were dialyzed against 10 mM Tris/HCl, pH8.0 and aliquots were examined by starch gel electrophoresis. Peaks (I), (II) and (III) contained GST-5 and GST-1 isozymes and Peak (IV) contained mainly GST-3. Peak (V) contained mainly GST-6. Therefore Peaks (I), (II) and (III) containing GST-5 were pooled and dialyzed against 20 mM phosphate buffer (pH6.5).

Step 3. The dialyzed sample (83 ml) was applied to a column of CM-Sephadex (3.5 cm x 30 cm) equilibrated in 20 mM phosphate buffer (pH6.5). GST-5 did not bind to the CM-Sephadex under these conditions and the active fractions which passed through the column were pooled with a final volume of 470 ml. Application of KCl up to 0.3 M did not elute any additional GST-5.

Step 4. Solid ammonium sulfate was added to the pooled sample to 40% saturation and the mixture was gently stirred overnight. The precipitate was collected by centrifugation at 10,000 x g for 30 minutes. The precipitate
was dissolved with an adequate volume of 10 mM Tris/HCl buffer (pH8.0) and dialyzed against the same buffer (pH8.0). The sample (35 ml) was applied to a column of DEAE cellulose (3.5 cm x 30 cm) (II) equilibrated with 10 mM Tris/HCl buffer, pH8.0 and extensively washed with the Tris/HCl buffer until enzyme activity and protein were no longer detected in the eluant. A 1 litre linear gradient between 0 and 0.05 M KCl in 10 mM Tris/HCl buffer (pH8.0) was applied to the column, and then followed with another between 0.05 and 0.1 M KCl. Each active fraction was tested by starch gel electrophoresis to determine if GST-5 was present. The active fractions were pooled in three groups. Pool (I) tube No.241-256 (91 ml), Pool (II) tube No.257-301 (250 ml) and Pool (III) No.305-321 (115 ml). Pool (I) contained only GST-5 and Pools (II) and (III) contained mainly GST-1.

Step 5. The sample was concentrated by ultrafiltration (Amicon DIAFLO P10) and dialyzed against 20 mM phosphate buffer (pH7.0). The dialyzed sample was applied to a column (2.3 cm x 6 cm) of GSH-agarose (pH7.0) and washed extensively. GST-5 was eluted from the column with 50 mM Tris (pH9.6) containing 5 mM GSH.

The detailed procedures for evaluation of substrate affinity, kinetic analysis, inhibition studies, heat stability, pH optimum and immunodiffusion using antisera raised against GSTs-1, 2 and 3 have been described in Chapter 3.
RESULTS

Purification of GST-5 from human brain: A summary of the purification is shown in Table VII-1. The elution patterns for the purification of GST-5 are shown in Figures VII-1, 2 and 3. GST-5 was separated from GST-1 on the second DEAE cellulose column (pH 8.0) after chromatography on DEAE cellulose and CM-Sephadex (pH 6.5) was used to remove other proteins from the sample.

Subunit molecular weight: A single protein was identified by SDS/polyacrylamide gel electrophoresis after reduction of disulphide bonds at 100°C. The subunit molecular weight was found to be 27,500, identical to that of GST-1 (Figure VII-4).

Kinetic analysis, specific activity and inhibition studies: The $K_m$ values of the GST-5 isozyme are shown in Table VII-2.

Table VII-2. The $K_m$ values (mM) of the GST-5 isozyme

<table>
<thead>
<tr>
<th></th>
<th>GST-5</th>
<th>GST-1 a</th>
<th>GST-3 b</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>0.23</td>
<td>0.46</td>
<td>0.27</td>
</tr>
<tr>
<td>CDNB</td>
<td>0.23</td>
<td>0.75</td>
<td>0.17</td>
</tr>
</tbody>
</table>

a: the $K_m$ values of GST-1*1 derived from Chapter 3.
b: the data derived from Chapter 5.
Table VII-1. Purification of GST-5 from human brain

<table>
<thead>
<tr>
<th></th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Activity (U/ml)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>2410</td>
<td>14493.7</td>
<td>0.298</td>
<td>718.18</td>
<td>0.050</td>
<td>100</td>
</tr>
<tr>
<td>DEAE cellulose (I)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(I)</td>
<td>410</td>
<td>745.79</td>
<td>0.132</td>
<td>54.12</td>
<td>0.073</td>
<td>7.5</td>
</tr>
<tr>
<td>(II)</td>
<td>460</td>
<td>238.28</td>
<td>0.120</td>
<td>55.20</td>
<td>0.232</td>
<td>7.7</td>
</tr>
<tr>
<td>(III)</td>
<td>280</td>
<td>423.92</td>
<td>0.591</td>
<td>165.40</td>
<td>0.390</td>
<td>23.0</td>
</tr>
<tr>
<td>(VI)</td>
<td>285</td>
<td>485.64</td>
<td>0.578</td>
<td>164.73</td>
<td>0.339</td>
<td>22.9</td>
</tr>
<tr>
<td>(V)</td>
<td>925</td>
<td>1660.38</td>
<td>0.136</td>
<td>123.80</td>
<td>0.076</td>
<td>17.5</td>
</tr>
<tr>
<td>CM-Sephadex c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(I)</td>
<td>470</td>
<td>164.50</td>
<td>0.185</td>
<td>86.95</td>
<td>0.529</td>
<td>12.1</td>
</tr>
<tr>
<td>(II)</td>
<td>91</td>
<td>13.01</td>
<td>0.047</td>
<td>4.27</td>
<td>0.328</td>
<td>0.6</td>
</tr>
<tr>
<td>(III)</td>
<td>250</td>
<td>30.25</td>
<td>0.079</td>
<td>19.70</td>
<td>0.651</td>
<td>2.7</td>
</tr>
<tr>
<td>(IV)</td>
<td>115</td>
<td>12.77</td>
<td>0.103</td>
<td>11.83</td>
<td>0.926</td>
<td>1.6</td>
</tr>
<tr>
<td>GSH-agarose f</td>
<td>16</td>
<td>0.78</td>
<td>0.154</td>
<td>2.47</td>
<td>3.171</td>
<td>0.3</td>
</tr>
</tbody>
</table>

a: Protein concentration was determined by the method of Bradford (1976).
b: The enzyme activity was measured at 37°C and pH7.5 using 0.2 mM CDNB and 0.2 mM GSH as substrates. U = µM conjugate/minute.
c: The chromatography was carried out at pH8.0 and a linear KCl gradient was made. Peaks (I), (II) and (III) were used for the next step.
d: The chromatography was performed at pH6.5.
e: The chromatography was carried out at pH8.0 and a KCl linear gradient was made. Pool (I) was used for the next step.
f: The affinity chromatography was performed at pH7.0 and the active eluant was collected by application of 5 mM GSH (pH9.6).
Figure VII-1. Elution pattern of GST-5 and protein from DEAE cellulose (I) (pH 8.0).

Figure VII-2. Elution pattern of GST-5 and protein from DEAE cellulose (II) (pH 8.0).

Figure VII-3. Elution pattern of GST-5 and protein from GSH-agarose (pH 7.0).
Figure VII-4. SDS/polyacrylamide gel electrophoresis of purified GST-5.

Lane 1: GST-1 and GST-2
Lane 2: GST-5
Lane 3: Molecular weight markers
Table VII-3 summarizes the specific activity of GST-5 with several substrates. The activity with CDNB was relatively low and this isozyme did not have broad substrate specificity.

Table VII-3. Specific activity of GST-5 with different substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>GST-5 (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-chloro-2,4-dinitrobenzene</td>
<td>0.698</td>
</tr>
<tr>
<td>1,2-dichloro-4-nitrobenzene</td>
<td>0.0372</td>
</tr>
<tr>
<td>1,2-epoxy-3-(p-nitrophenoxy)propane</td>
<td>0</td>
</tr>
<tr>
<td>Ethacrynic acid</td>
<td>0</td>
</tr>
<tr>
<td>trans-4-phenyl-3-buten-2-one</td>
<td>0</td>
</tr>
<tr>
<td>Bromosulfophthalein</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\): \(\mu\)M/mg of protein/minute

GST-5 did not exhibit any glutathione peroxidase activity when cumen hydroperoxide, \(\text{-butyl hydroperoxide}\) and \(\text{H}_2\text{O}_2\) were used as substrates.

The inhibitory effects of five chemicals on the GST-5 isozyme are shown in Figure VII-5. The overall inhibitory profiles of GST-5 activity were similar to those of GST-1 activity except for cholate which did not inhibit up to 100 \(\mu\)M.
Table VII-3 summarizes the specific activity of GST-5 with several substrates. The activity with CDNB was relatively low and this isozyme did not have broad substrate specificity.

Table VII-3. Specific activity of GST-5 with different substrates

<table>
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</tr>
<tr>
<td>trans-4-phenyl-3-buten-2-one</td>
<td>0</td>
</tr>
<tr>
<td>bromosulfophthalein</td>
<td>0</td>
</tr>
</tbody>
</table>

a: µM/mg of protein/minute

GST-5 did not exhibit any glutathione peroxidase activity when cumene hydroperoxide, t-butyl hydroperoxide and \( \text{H}_2\text{O}_2 \) were used as substrates.

The inhibitory effects of five chemicals on the GST-5 isozyme are shown in Figure VII-5. The overall inhibitory profiles of GST-5 activity were similar to those of GST-1 activity except for cholate which did not inhibit up to 100 µM.
Figure VII-5. The inhibitory effect of some chemicals on GST-5 activity.

A: cholate
B: chenodeoxycholate
C: lithocholate 3-sulfate
D: ellagic acid
E: sulfobromophthalein
Cholate (µM)  

Chenodeoxycholate (µM)  

Ellagic acid (µM)  

Bromosulfophthalein (µM)

GST activity (% of control)
Figure VII-6. The effect of temperature on GST-5.

A. incubated for various times at 45°C.

B. incubated for 10 minutes at various temperatures.

Figure VII-7. The effect of pH on GST-5 activity.
A

% of activity at 0 min.

Time (minute)

B

% of activity at 4°C

Temperature (°C)

C

% of maximum

pH

5.5 6.5 7.5 8.5 9.5 10.5 11.5
Figure VII-8. Immunodiffusion of GST-5 against anti GST-1, anti GST-2 and anti GST-3.

A) anti GST-1 against GST-1*2 and GST-5
B) anti GST-2 against GST-2*2 and GST-5
C) anti GST-3 against GST-3 and GST-5
The heat stability profile of the GST-5 isozyme is illustrated in Figures VII-6A & 6B. The GST-5 isozyme was slightly more stable than the GST-1 isozymes when it was incubated at 45 °C for 10-30 minutes.

The pH curve of GST-5 is shown in Figure VII-7. The pH optima for the GST-5 and GST-1 isozymes are essentially the same falling between pH7.0 and pH8.0.

Immunological studies: As shown in Figure VII-8, antibodies raised against GST-1 were found to cross-react with GST-5. However, antibodies raised against GST-2 and GST-3 failed to cross-react.

DISCUSSION

The data presented in Chapter 2 show that GST-5 seems to be a brain specific glutathione S-transferase isozyme which is in partial agreement with similar observations made by Laisney et al. (1984).

Three glutathione S-transferase isozymes were purified from brain by Theodore et al. (1985). They reported that there were cationic (pI=8.3) and anionic (pI=5.5 and 4.6) forms in the brain they studied. It is difficult to relate these reported forms to the isozymes that can be detected by electrophoresis and specific staining. Because of the polymorphism at the GST-1 locus, the presence of brain specific GST-5 and the number of isozymes with acidic isoelectric points will vary from sample to sample. Since the
pI 5.5 isozyme reported by Theodore et al. (1985) did not cross-react with antiserum against the isozymes from placenta and lung, it would appear that their isozyme is not GST-3 which is the predominant form in those tissues. It seems likely that the isozyme reported to have a pI of 5.5 may correspond to either GST-1 or GST-5 or possibly both isozymes.

Comparisons of the properties of GST-5 and GST-1 clearly show many similarities. However, $K_m$ values for CDNB and GSH obtained for GST-5 were markedly lower than those obtained for GST-1 (Table VII-2). Since a single protein band was observed after reduction and SDS/polyacrylamide gel electrophoresis, it seems most unlikely that GST-5 is comprised of two different subunits. Immunodiffusion tests clearly indicated the immunological similarity of GST-1 and GST-5. In contrast antisera directed against GST-2 and GST-3 did not cross-react with GST-5. While it is possible that the GST-5 preparation may have a minor GST-1 contamination, this is unlikely to be of sufficient magnitude to create a significant precipitin band during immunodiffusion since no GST-1 activity could be detected after starch gel electrophoresis and specific staining for GST activity.

Overall, the data suggest that GST-5 is a product of the GST-1 gene which undergoes brain specific post-translational modification that results in a change in electrophoretic mobility. While it remains possible that GST-5 is
the product of an independent gene, that gene must have a product that is very similar to GST-1. Further study of the amino acid sequence of the GST-1 and GST-5 isozymes is required to reach a final conclusion on this point.

The ability of extrahepatic organs to metabolize drugs and the consequences of these actions have recently been of interest in pharmacology and toxicology. The brain, the most intricate and delicate of the major organs, also has the capacity to detoxify xenobiotics. The cerebral metabolism of such compounds may play important roles in both causing disruption of neural function and in contributing to drug action. Because of the limited regenerative ability of brain, an understanding of its mechanisms for the detoxification of xenobiotics is of particular importance in man. Although the glutathione S-transferases are clearly present in brain tissue and may be involved in xenobiotic metabolism, their significance in brain metabolism is clearly not yet known.

As described in Chapter 1, a glutathione S-transferase may catalyze the reaction to form leukotriene C₄ (LTC₄). Lindgren et al. (1984) isolated LTC₄, LTD₄, and LTE₄ from rat brain incubations and demonstrated the regional distribution of LTC₄ biosynthesis in the brain. These results may suggest a potential role of the leukotrienes in brain function and one of the glutathione S-transferase isozymes may regulate LTC₄ biosynthesis in this tissue.
Chapter 8

The purification and biochemical characterization of the GST-6 isozyme from human brain

MATERIALS AND METHODS

Materials, and the detailed procedures for the examination of glutathione S-transferase activity, protein concentration, starch gel electrophoresis, and SDS/polyacrylamide gel electrophoresis have been described in Chapter 7 and 8.
INTRODUCTION

GST-6 was the most anodal of all the isozymes detected in the experiments described in Chapter 2. This isozyme was found to be expressed in every tissue except erythrocytes (see Figure II-2).

The presence of the GST-6 isozyme in human tissues has not been previously described by other researchers. It is possible that the GST-6 isozyme is a post-translationally modified form of GST-3. According to Harris (1980), deamidation is a frequent post-translational modification of enzymes. The removal of amide groups from asparagine and glutamine residues on the outer surface of enzyme molecules would be expected to increase their net negative charge and hence their anodal electrophoretic mobility.

To determine if GST-6 is an independent gene product or if it is derived from GST-3, it has been purified from the same brain sample as GST-5 was purified from and reported in Chapter 7.

MATERIALS AND METHODS

Materials, and the detailed procedures for the determination of glutathione S-transferase activity, protein concentration, starch gel electrophoresis, and SDS/polyacrylamide gel electrophoresis have been described in Chapters 2 and 3.
Purification of GST-6 from human brain:

Step 1 and Step 2. The first stage of the purification of GST-6 from human brain was the same with Steps 1 and 2 of the purification of the GST-5 isozyme (see Chapter 7).

Step 3. Peak V (925 ml), which was eluted from DEAE cellulose column (1), was concentrated by ultrafiltration and extensively dialyzed against 20 mM phosphate buffer (pH7.0). The dialyzed sample (130 ml) was applied to an Affi-Gel Blue column (2.3 cm x 14 cm) equilibrated in 20 mM phosphate buffer, pH7.0. After washing extensively with the phosphate buffer, 0.5 M KCl in 20 mM phosphate buffer (pH7.0) was applied to the column. After adding KCl, the active fractions were collected and their pooled volume was 220 ml.

Step 4. The sample was again concentrated by ultrafiltration and dialyzed against 20 mM phosphate buffer (pH7.0). The dialyzed sample (18 ml) was applied to a column (2.3 cm x 6 cm) of GSH-agarose (pH7.0). The column was extensively washed with 20 mM phosphate buffer, pH7.0 and then the purified GST-6 was eluted with 50 mM Tris (pH9.6) containing 5 mM GSH.

The detailed procedures for substrate affinity, kinetic analysis, inhibition studies, heat stability, pH optimum and immunodiffusion using antisera raised against GSTs-1, 2 and 3 have been described in Chapter 3.
RESULTS

Purification of GST-6 from human brain: A summary of the purification is shown in Table VIII-1. The elution patterns for the purification of GST-6 are shown in Figures VIII-1 and 2. GST-6 was separated from less anionic glutathione S-transferase isozymes by chromatography on DEAE cellulose column (I) (pH8.0). The purified GST-6 clearly had increased electrophoretic mobility when compared with GST-3 from lung (Figure VIII-3).

Subunit molecular weight: Two different subunits were identified by SDS/polyacrylamide gel electrophoresis after reduction of disulphide bonds at 100°C. Their molecular weights were 27,500 and 26,950. As shown in Figure VIII-4, the heavier subunit of GST-6 and GST-1 appear to be of similar size.

Kinetic analysis, specific activity and inhibition studies: The $K_m$ values for GSH and CDNB are shown in Table VIII-2 in comparison with the values obtained for GST-3 in Chapter 5.

Table VIII-2. The $K_m$ values (mM) for the GST-6 isozyme

<table>
<thead>
<tr>
<th></th>
<th>GST-6</th>
<th>GST-3 a</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>0.18</td>
<td>0.27</td>
</tr>
<tr>
<td>CDNB</td>
<td>0.56</td>
<td>0.17</td>
</tr>
</tbody>
</table>

a: the data derived from Chapter 5.
Table VIII-1. Purification of GST-6 from human brain

<table>
<thead>
<tr>
<th></th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Activityb (U/ml)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>2410</td>
<td>14493.7</td>
<td>0.298</td>
<td>718.18</td>
<td>0.050</td>
<td>100</td>
</tr>
<tr>
<td>DEAE cellulose (I)c</td>
<td>(V) 925</td>
<td>1660.38</td>
<td>0.136</td>
<td>123.80</td>
<td>0.076</td>
<td>17.5</td>
</tr>
<tr>
<td>Affi-Gel Blue d</td>
<td>220</td>
<td>370.04</td>
<td>0.146</td>
<td>32.32</td>
<td>0.087</td>
<td>4.5</td>
</tr>
<tr>
<td>GSH-agarose e</td>
<td>39</td>
<td>1.27</td>
<td>0.121</td>
<td>4.72</td>
<td>3.880</td>
<td>0.71</td>
</tr>
</tbody>
</table>

a: Protein concentration was determined by the method of Bradford (1976).
b: The enzyme activity was measured at 37°C and pH7.5 using 0.2 mM CDNB and 0.2 mM GSH as substrates. U = µM conjugate/minute.
c: The chromatography was carried out at pH8.0 and a KCl linear gradient was applied.
d: The chromatography was carried out at pH7.0 and 0.5 M KCl was applied.
e: The affinity chromatography was performed at pH7.0 and the active eluant was collected by application of 5 mM GSH (pH9.6).
Figure VIII-1. Elution pattern of GST-6 and protein from Affi-Gel Blue (pH7.0).

Figure VIII-2. Elution pattern of GST-6 and protein from GSH-agarose (pH7.0).
Figure VIII-3. Starch gel electrophoresis of purified GST-6
Lane 1: Purified GST-6
Lane 2: GST-3 from a crude lung extract
Lane 3: Purified GST-3

Figure VIII-4. SDS/polyacrylamide gel electrophoresis of purified GST-6
Lane 1: GST-6
Lane 2: GST-3
Lane 3: Molecular weight markers
Table VIII-3 summarizes the specific activity of GST-6 with several substrates. The activity with CDNB was relatively low and this isozyme did not have broad substrate specificity.

Table VIII-3. Specific activity of GST-6 with various substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>GST-6 a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-chloro-2,4-dinitrobenzene</td>
<td>9.000</td>
</tr>
<tr>
<td>1,2-dichloro-4-nitrobenzene</td>
<td>0.4078</td>
</tr>
<tr>
<td>1,2-epoxy-3-(p-nitrophenoxy)propane</td>
<td>0</td>
</tr>
<tr>
<td>ethacrynic acid</td>
<td>0</td>
</tr>
<tr>
<td>trans-4-phenyl-3-buten-2-one</td>
<td>0</td>
</tr>
<tr>
<td>bromosulfophthalein</td>
<td>0</td>
</tr>
</tbody>
</table>

a: µM/mg of protein/minute

GST-6 did not exhibit any glutathione peroxidase activity when cumene hydroperoxide, 1-butyl hydroperoxide and \( \text{H}_2\text{O}_2 \) were used as substrates.

The inhibitory effects of five chemicals on the GST-6 isozyme are shown in Figure VIII-5. Ellagic acid and bromosulfophthalein were found to be much stronger inhibitors of GST-6 than GST-3.

The heat stability profiles of the GST-6 isozyme are illustrated in Figures VIII-6A & B. The heat stability of
Figure VIII-5. The inhibitory effect of some chemicals on GST-6 activity.

A: cholate
B: chenodeoxycholate
C: lithocholate 3-sulfate
D: ellagic acid
E: sulfobromophthalein
Figure VIII-6. The effect of temperature on GST-6.

A. incubated for various times at 45°C.
B. incubated for 10 minutes at various temperatures.

Figure VIII-7. The effect of pH on GST-6 activity.
Figure VIII-8. Immunodiffusion of GST-6 against anti GST-1, anti GST-2 and anti GST-3.

A) anti GST-1 against GSTs-1*1, 4, 5 and 6

B) anti GST-2 against GST-2*2 and GST-6

C) anti GST-3 against GST-3 and GST-6
both GST-6 and GST-3 appear to be essentially the same.

The pH curve for the GST-6 isozyme is shown in Figure VIII-7. The pH optimum for GST-6 was between pH7.0 and pH8.0 the same as GST-3.

Immunological studies: Antibodies raised against GST-1*2, GST-2*2 and GST-3 failed to provide any evidence of cross-reaction with GST-6 (Figure VIII-8).

DISCUSSION

GST-6, which was detected in all human tissues, except erythrocytes (see Chapter 2), was purified in the present experiments.

Because two protein bands of highly purified GST-6 (subunit molecular weight: 27,500 and 26,950) were observed after SDS/polyacrylamide gel electrophoresis, it seems likely that GST-6 is composed of two dissimilar subunits. The most acidic isozyme purified from brain by Theodore et al. (1985) was also reported to be a heterodimer. That isozyme was found to cross react with antibodies raised against glutathione S-transferases purified from lung and placenta. This suggests that the pI4.6 isozyme purified by Theodore et al. (1985) may share a subunit with GST-3. Both the subunits derived from GST-6 appear to be larger than the single subunit derived from GST-3 (25,100) in the present studies. In addition, antibodies raised against GST-3
failed to cross-react with GST-6. These observations largely exclude the possibility that GST-6 is related to GST-3.

The $K_m$ value of GST-6 for GSH is much lower than that of GST-1, on the other hand, the $K_m$ value of GST-6 for CDNB is the highest of all the glutathione S-transferase isozymes purified in the present experiments. The inhibition studies showed that there were clear differences between GST-1 and GST-6, namely, chenodeoxycholate inhibited the GST-1 activity but not the GST-6 activity. Furthermore, cholate did not significantly inhibit GST-6 activity, but markedly inhibited the GST-1 activity at higher concentrations. These biochemical characteristics indicate clear differences between GST-1 and GST-6.

When GST-2 and GST-6 are compared, they can readily be distinguished by the glutathione peroxidase activity of GST-2. Because GST-6 is expressed in all tissues studied except erythrocytes, and GST-4 and GST-5 are highly tissue specific, it seems unlikely that GST-6 is related to these isozymes. Furthermore, the findings of Chapter 7 suggest that GST-5 is a modified form of GST-1 and can be discriminated from GST-6 by its inhibition by chenodeoxycholate and its $K_m$ for CDNB.

Although the molecular weights of GST-6 subunits are similar to those of GST-1 and GST-2, antibodies raised against GST-1*2 and GST-2*2 failed to cross-react with GST-6.
It therefore seems unlikely that GST-6 shares subunits with any of the other commonly expressed glutathione S-transferase isozymes.

Since GST-6 appears to be composed of two dissimilar subunits, it is possible that GST-6 is the product of two separate genes which for convenience could be termed GST-6H and GST-6L for the heavy and light subunits respectively. It is not clear at this stage just which isozyme corresponds to the acidic form (pI 4.4) purified by Theodore et al. (1985) and it remains a possibility that those authors have co-purified GST-3 and potentially GST-6.

In conclusion, the present experiments suggest that GST-6 is not a modified product of either GST-3 or the other glutathione S-transferase genes reported in the previous chapters, but the product of independent genes.
Chapter 9

General Discussion

The coexistence of multiple forms of glutathione S-transferase has been the common finding of all the studies made in a variety of species. The heterogeneity of the glutathione S-transferase is not unique but reflects the general variation in other species. In rats, the most extensively investigated species, some progress has been made toward an understanding of the relationships between the different glutathione S-transferases enzymes (Mannervick, 1983). Some rat glutathione S-transferase isozymes result from hybrid dimeric combination of genetically distinct subunits, thus the capacity to form hybrid dimers has substantially increased the apparent heterogeneity of the rat glutathione S-transferases. Bord (1960) showed the electrophoretic pattern of rat glutathione S-transferase in liver, and his results suggested that starch gel electrophoresis may be a useful technique for the study of the heterogeneity of this enzyme. Subsequently, Marunaka and Gassar (1981) reported electrophoretic variation of glutathione S-transferase in one of 22 inbred rat strains studied. Unfortunately, Marunaka and Gassar (1981) were not able to relate the variant isozyme to the isozyme that had been previously identified by chromatographic techniques. While most studies have considered the glutathione S-transferases in rat liver, few studies of other tissues have revealed additional isozyme forms (Mannervick, 1983).
The coexistence of multiple forms of glutathione S-transferase has been the common finding of all the studies made in a variety of species. The heterogeneity of the glutathione S-transferase in man is, therefore, not unique but reflects the generalized situation in other species. In rats, the most extensively investigated species, some progress has been made towards an understanding of the relationships between the different glutathione S-transferase isozymes (Mannervick, 1985). Some rat glutathione S-transferase isozymes result from hybrid dimeric combination of genetically distinct subunits, thus the capacity to form hybrid dimers has substantially increased the apparent heterogeneity of the rat glutathione S-transferases. Board (1980) showed the electrophoretic pattern of rat glutathione S-transferases in liver, and his results suggested that starch gel electrophoresis may be a useful technique for the study of the heterogeneity of this enzyme. Subsequently, Matsumoto and Gasser (1983) reported electrophoretic variation of glutathione S-transferase in one of 22 inbred rat strains studied. Unfortunately, Matsumoto and Gasser (1983) were not able to relate the variant isozyme to the isozymes that had been previously identified by chromatographic techniques. While most studies have considered the glutathione S-transferases in rat liver, the few studies of other tissues have revealed additional isozyme forms (Mannervick, 1985).
As reviewed in Chapter 1, multiple forms of glutathione S-transferase have been reported in man. These reports have ranged from the description of multiple chromatographically fractionated isozymes from a single liver sample (Kamisaka, 1975) to electrophoretically fractionated isozymes from a large number of individuals (Board, 1981a). The report of Board (1981a) provided an initial genetic model to explain the relationship between the isozymes observed after the electrophoresis of liver and erythrocyte extracts. However, because in practice, it is impossible to formally test Board's model in man, some doubt about its validity remained. Therefore, it was the aim of the present studies to further examine Board's model by investigating the distribution of glutathione S-transferase isozymes in additional tissues, and to characterize and compare their properties. Where possible, it was hoped to identify the isozymes characterized by others in terms of the genetic nomenclature proposed originally by Board (1981a).

During the course of this study, several other investigations, notably, Strange et al. (1984b), Laisney et al. (1984) and Stockman et al. (1985) have also investigated aspects of this problem and made contributions to the understanding of this field. Where they are relevant, the findings of these authors have been discussed in detail.

Each experimental chapter has been provided with an independent discussion section, and the significance of many individual details have been discussed in those chap-
Chapter 9. General discussion

ters. However, the interrelationship among the six isozymes have been only partially considered. Consequently, some results relevant to the potential interrelationships are summarized and discussed further here. To facilitate comparison, some of the data obtained for each isozyme are summarized in Table IX-1.

Table IX-1. Comparison of human glutathione S-transferase isozymes

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Subunit</th>
<th>M.W.</th>
<th>(K_m) (GSH) &amp; (CDNB)</th>
<th>*</th>
<th>**</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-1</td>
<td>1*1</td>
<td>27,500</td>
<td>0.750</td>
<td>0.460</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>1*2</td>
<td>27,500</td>
<td>0.610</td>
<td>0.460</td>
<td>a</td>
</tr>
<tr>
<td>GST-2</td>
<td>2*1</td>
<td>26,900</td>
<td>0.153</td>
<td>0.067</td>
<td>b GSH-Px (+)</td>
</tr>
<tr>
<td></td>
<td>2*2</td>
<td>26,900</td>
<td>0.144</td>
<td>0.071</td>
<td>b GSH-Px (+)</td>
</tr>
<tr>
<td>GST-3</td>
<td></td>
<td>25,100</td>
<td>0.270</td>
<td>0.170</td>
<td>c</td>
</tr>
<tr>
<td>GST-4</td>
<td></td>
<td>27,300</td>
<td>0.154</td>
<td>0.333</td>
<td>d</td>
</tr>
<tr>
<td>GST-5</td>
<td></td>
<td>27,500</td>
<td>0.224</td>
<td>0.226</td>
<td>a</td>
</tr>
<tr>
<td>GST-6</td>
<td></td>
<td>27,500 &amp; 26,950</td>
<td>0.178</td>
<td>0.568</td>
<td>d</td>
</tr>
</tbody>
</table>

* Immunological studies
  a: cross-reacts only with anti GST-1
  b: cross-reacts only with anti GST-2
  c: cross-reacts only with anti GST-3
  d: does not cross-react with anti GSTs-1, 2 or 3

** glutathione peroxidase activity
There are several mechanisms by which the heterogeneity of the glutathione S-transferases could arise. These include:

i) the presence of a number of genes producing different subunits of the enzyme

ii) the presence of allelic variants with small charge changes

iii) post-translational modifications.

These three factors can potentially interact to produce additional heterogeneity.

The origin of most heterogeneity is likely to be the presence of multiple genes which code for a number of different subunits. As has been noted earlier, the rat liver glutathione S-transferases are the products of multiple genes, which produce several subunits that appear to be able to form homo- and hetero-dimers, and thereby creating a high degree of heterogeneity (Mannervick, 1985). Tissue specificity and differential induction can also be expected to influence the number of genes that are active, and thereby the number of isozymes that are present.

Allelic variations, possibly resulting from point mutations in glutathione S-transferase subunit genes can also be expected to contribute to the overall heterogeneity and will be responsible for some individual variations.

Post-translational modifications can involve several mechanisms including:
1) acetylation
2) addition of phosphate groups
3) addition or removal of carbohydrate chains
4) proteolytic processing
5) aggregation and polymerization
6) oxidation of sulphydryls
7) deamidation (Harris, 1980).

In addition, since glutathione S-transferase is known to be a binding protein (see Chapter 1), it is possible that the binding of certain metabolites or xenobiotic compounds may create what appears to be additional isomers. Many post-translational events could be tissue specific. Similarly, since the rat glutathione S-transferases are known to be inducible (Chasseaud, 1979), post-translational modification could also be inducible and result from environmental influences. This latter possibility could give rise to significant individual differences.

The extent to which these factors are responsible for the heterogeneity of the human glutathione S-transferases can be evaluated in part from many of the results obtained in the present investigations.

**GST-1 isozymes**

As discussed previously in Chapter 1, Board (1981a) suggested that the glutathione S-transferase isozymes expressed in liver and erythrocytes are the products of three separate genes (GST-1, GST-2 and GST-3). Up to three
isozymes were reported to be the products of the GST-1 locus and were thought to result from the homo- and heterodimeric combination of two allelic forms with differing electrophoretic mobility. In the present study, both apparently homozygous forms of GST-1 were purified and compared. Both biochemical and immunological comparisons confirmed the identity of these two isozymes and fully supported the original prediction of Board (1981a) that GST-1*1 and GST-1*2 are the products of alleles at a single locus. The additional electrophoretic studies of Strange et al. (1984b) and Laisney et al. (1984) have also confirmed this relationship.

The presence of a common null allele at the GST-1 locus was also predicted by Board (1981a) and the findings of several other groups summarized in Table 1-5 (page 23), have also confirmed Board's observations. The occurrence of the null allele is of particular importance when an attempt is made to identify the isozymes biochemically characterized by others. Because of differences between laboratories, for example, in the source of molecular weight standards, the values obtained for some biochemical characteristics seem to vary. Therefore, where the differences between different gene products may only be small, the comparison of their characteristics reported by different laboratories is difficult. Warholm et al. (1981b; 1983) have reported a 'near-neutral' glutathione S-transferase isozyme which appears to have biochemical characteristics similar to
those obtained for GST-1 in Chapter 3. Since Warholm et al. (1983) have also reported that there is a common deficiency of the 'near-neutral' isozyme which they characterized, it can be strongly argued that this isozyme is a product of GST-1 locus and identical to one of the GST-1 isozymes studied here.

**GST-2 isozymes**

Kamisaka et al. (1975) purified a number of cationic isozymes of glutathione S-transferase from liver and concluded that these isozymes were due to post-translational modification of a single gene product. In contrast, the results of Stockman et al. (1985) have suggested that the cationic glutathione S-transferases may be the product of multiple genes. Until the present investigation, there was little evidence to confirm the identity of the GST-2 isozymes reported by Board (1981a) and the cationic isozymes reported by several groups.

The data presented and discussed in Chapter 4 suggest that the cationic isozymes are equivalent to the electrophoretically cathodal isozymes reported to be the products of the GST-2 locus by Board (1981a). One distinctive feature of the GST-2 isozymes was their glutathione peroxidase activity which was not a property of any of the other glutathione S-transferase isozymes characterized in this study. As noted in Chapter 4, glutathione peroxidase activity was a property of the cationic isozymes character-
ized by Awasthi et al. (1980). The heterogeneity of the cationic glutathione S-transferases (GST-2) probably results from the post-translational modification of a single gene product but not allelic variation as proposed by Board (1981a) or multiple genes inferred by Stockman et al. (1985).

Some additional protein chemistry studies were undertaken to obtain further evidence of the interrelationships of the glutathione S-transferases (see Appendix II, pp. 188-197). Due to technical difficulties, very little reliable data was obtained, however one point warrants further discussion here. A sample containing both the GST-2*1 and GST-2*2 isozymes was subjected to automated N-terminal sequence analysis. However, no sequence has obtained because the amino terminal was blocked. Since this sample contained both GST-2*1 and GST-2*2 isozymes, it can be concluded that the amino terminal of both isozymes must be blocked. This finding is in agreement with the recent report of Alin et al. (1985) who reported the amino terminal of human cationic glutathione S-transferase was blocked. Since Alin et al. (1985) were able to obtain the N-terminal sequences for other near-neutral and acidic glutathione S-transferase isozymes, it seems likely that a blocked N-terminal is a particular characteristic of the cationic or GST-2 isozymes and does not exclude the conclusion that they are the product of a single gene.
GST-3 isozyme

The third locus proposed by Board (1981a) was GST-3 and it was shown to be expressed in erythrocytes. Subsequently, other studies including Chapter 2, Strange et al. (1984b) and Laisney et al. (1984) have shown that an isozyme with similar electrophoretic mobility was found in most tissues. In many tissues, including lung, erythrocytes and lymphocytes, the major isozyme expressed appears to be GST-3, although it has been termed "ρ" and "π" by other investigators. The identity of the GST-3 isozyme from several tissues has been confirmed by several independent studies. Marcus et al. (1977) originally termed the isozyme which they studied in erythrocytes as isozyme "ρ". Guthenberg and Mannervick (1981) suggested that isozyme "π" from placenta is identical or closely related to isozyme "ρ" from erythrocytes. In addition Dao et al. (1984) concluded that the anionic (or acidic) isozymes from lung and placenta were identical. Taken together these studies confirm that all these tissues express an identical isozyme. In the present studies antibodies raised against GST-3 purified from lung did not cross-react with any of the other purified isozymes confirming that GST-3 is a distinct gene product. This conclusion has also been confirmed recently by the determination of the N-terminal amino acid sequence for an acidic isozyme "π" which differed from that determined for a near-neutral isozyme "μ" or GST-1 (Alin et al., 1985).
GST-4 isozyme

The survey of different tissues carried out in Chapter 2 revealed an isozyme that appeared to be restricted to muscle tissue. While Laisney et al. (1984) also reported an additional isozyme in muscle, it was not clear if the isozyme was the product of an additional gene. The biochemical and immunological studies presented in Chapter 6 indicate that GST-4 is the product of an independent gene and is not closely related to GST-1, GST-2 or GST-3.

GST-5 isozyme

An isozyme termed GST-5 was identified specifically in brain tissue in Chapter 2 and it was purified and studied further in Chapter 7. This isozyme did not closely correspond with any of the isozymes previously purified from human brain by Theodore et al. (1985). However, comparison of its properties with the other isozymes purified in this study indicated that GST-5 may be a form of GST-1 that has undergone brain-specific post-translational modification. All of the individuals who expressed GST-1 and whose brain tissues were studied in Chapter 2 were of the GST-1*1 phenotype. Additional studies are needed to determine if individuals with the GST-1*2 or heterozygous GST-1*2-1 phenotypes also have additional brain specific isozymes with the appropriate differences in electrophoretic mobility.
GST-6 isozyme

Every tissue studied in Chapter 2, with the exception of erythrocytes, expressed an acidic isozyme which was termed GST-6. Variable levels of expression were found between tissues, and GST-6 was not observed in samples from some individuals. The purification of GST-6 was discussed in Chapter 8 and it is clear that GST-6 has a number of unique characteristics, in particular, its composition of two different subunits. Biochemical and immunological comparison of GST-6 with the other glutathione S-transferase isozymes indicated that it was probably not the result of post-translational modification or the recombination of subunits derived from the GST-1, GST-2 and GST-3 genes. It is therefore necessary to suggest that GST-6 is the product of two additional genes.

General aspects

The studies described here have shown that the heterogeneity of the human glutathione S-transferase is determined by a number of factors. Evidence which has been presented indicates that there are at least six different genes coding for the human glutathione S-transferases (GST-1, GST-2, GST-3, GST-4, GST-6H and GST-6L). These genes are variably expressed in different tissues, with GST-4 being specifically confined to muscle. Some variation appears to result from allelic polymorphism. The data obtained here and published by others support the original proposal of
Board (1981a) that the GST-1 locus is polymorphic and has three alleles including a "null". In contrast, the heterogeneity of the GST-2 or cationic isozymes may result from the post-translational modification of a single gene product. Some evidence was found for tissue-specific post-translational modification. The electrophoretically distinct isozyme termed GST-5 was only found in brain, but was found to be biochemically and immunologically similar to GST-1. It, therefore, seems likely that GST-5 is a brain specific, post-translationally modified product of the GST-1 gene.

In rats, there is considerable evidence for the generation of glutathione S-transferase isozyme heterogeneity by the formation of hetero-dimers between different subunits. The data obtained in the present studies suggest that this does not appear to be a major feature of the human isozymes. The data suggest that GST-6 is the product of two different subunits, however, these subunits do not appear to be components of the other isozymes studied here. Laisney et al. (1984) presented some electrophoretic evidence that the subunits of the muscle-specific isozyme they observed could form hetero-dimers with GST-1 subunits. The GST-4 purified here, contained subunits of a single size but the data presented here do not exclude the possibility that GST-1/GST-4 hetero-dimers may form in some individuals. In addition, Theodore et al. (1985) have reported human glutathione S-transferase isozymes that appear to be
composed of different subunits. Considerable care should be taken before attributing different subunits to a particular isozyme since the inadvertent co-purification of two isozymes with similar isoelectric points could potentially give misleading results. In the present studies, the relatively rapid electrophoretic separation of the purified isozymes alongside fresh tissue extracts permitted an additional check on the composition of the isozyme preparations being evaluated.

Although there are several potential roles for the glutathione S-transferases, including detoxification, leukotriene synthesis, heme transport and organic anion binding, there is as yet little evidence to indicate if particular isozymes have specialized functions. The tissue specificity of some isozymes suggests that those isozymes may have specialized functions, however, further investigations are needed to gain a better understanding of this point. As yet, no particular clinical abnormalities have been shown to be entirely due to deficiencies of glutathione S-transferase. This may be due to the fact that the presence of multiple genes and probably overlapping functional specificity make it unlikely that complete glutathione S-transferase deficiencies would ever occur in man. While it is possible that partial deficiencies of glutathione S-transferase activity such as homozygosity for the GST-1*1 allele may predispose individuals to greater risk of disorders such as neonatal jaundice (Board, 1981b), the
necessity to obtain tissue samples from major organs for diagnosis has so far prevented a proper evaluation of the effects of partial GST deficiency. In addition, any serious investigations of the effects of glutathione S-transferase deficiency can only be undertaken when the genetic and environmental factors affecting glutathione S-transferase activity are understood. The studies described here have confirmed and extended understanding of the genetic interrelationships of the human glutathione S-transferase isozymes. With this knowledge, further investigations using recombinant DNA techniques will hopefully permit the identification of deficient individuals from peripheral leukocyte DNA and facilitate the evaluation of the clinical significance of glutathione S-transferase deficiency.
BIBLIOGRAPHY


Glutathione-S-Transferase Gene Mapped to Chromosome 11 Is GST3 Not GST1

LETTER

The glutathione S transferases (GST) are an important family of isozymes which are responsible for the detoxification and elimination of a wide variety of potential carcinogens (1). The first investigation of GST from human liver utilized ion exchange chromatographic techniques and revealed the presence of numerous GST isozymes which were identified alphabetically by Greek symbols (2). In our laboratory, subsequent studies of samples from a large number of individuals by starch gel electrophoresis indicated that the GST isozymes in human liver are the product of at least three loci (3). Because of the association of some GST isozymes, a new numerical nomenclature was devised which reflects the genetic interrelationship between the different isozymes and can be expanded to account for any new genetic variants which may be discovered (3).

A recent study which electrophoretically analyzed the GST isozymes in man-mouse somatic cell hybrids suggested that the GST1 locus is located on chromosome 11 (4). While the data presented clearly indicated that a GST locus is located on chromosome 11, we believe that this locus is, in fact, GST3 and not the locus originally described as GST1.

In a Tris-EDTA-borate, pH 8.6, buffer system GST1 products migrate towards the anode between the origin and hemoglobin A, GST 2 products migrate towards the cathode, and the product of GST 3 migrates rapidly towards the anode in front of hemoglobin A (see Fig. 1). The data presented by Silberstein and Shows (4) clearly indicate that the gene coding for an electrophoretically fast GST isozyme which migrates in a position anodal to hemoglobin in Tris-EDTA-borate buffer, pH 8.6, is located on chromosome 11. The figures presented in that report do not show GST1 activity in any of the tissues used. It must be presumed that the individual from whom those tissues were obtained was homozygous for the null allele at the GST1 locus. This is not unexpected, as the null allele has a gene frequency exceeding 0.55 in all racial groups studied so far (3, 5).

It appears that, in the absence of GST1 products in the samples they studied, Silberstein and Shows (4) have mistaken the GST3 product for one of the GST1 isozymes. Our interpretation of the available data suggests

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1Received November 8, 1983.
that the locus assigned to chromosome 11 is actually GST3.

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LITERATURE CITED


REPLY

The question concerning which of the glutathione-S-transferase (GST) isozymes is encoded by a gene on human chromosome 11 has been resolved. This question is drawn to our attention here by Suzuki and Board. Previously, Board (1, 2) published a starch-gel electrophoretic procedure for separating several human GST isozymes. In 1982, we used a modification of this procedure to resolve GST isozymes in human-mouse somatic cell hybrids and came to the conclusion that the gene coding for the human GST1 isozyme is located on human chromosome 11 (3). Laisney et al. (4) have recently reported that the isozyme in question was not GST1 but another GST isozyme, GST3. They confirmed that the gene coding for the GST isozyme is, in fact, located on chromosome 11 by somatic cell hybrid gene mapping methods. Suzuki and Board (above) also present evidence that the isozyme is GST3 and not GST1.

Prompted by these reports, we have reexamined our findings and have found that the isozyme we identified as GST1 is, in fact, GST3. It seems that there were two reasons for us misidentifying GST3: (1) Most of our control samples were homozygous for the null allele of GST1, previously reported by Board (2) to be very common, and (2) the procedure we used to resolve human from mouse GST isozymes separated GST1 from mouse forms but was not optimal for GST1 activity. Because of a combination of the above, the prominent isozyme we scored as GST1 in our tissue and fibroblast samples was, in fact, GST3. Therefore, in Fig. 1 of Silberstein and Shows (3) GST1 should be labeled GST3. Thus, Laisney et al. (4) and Suzuki and Board (above) have correctly identified GST3, and it can be concluded that the human GST3 gene is confirmed to be located on chromosome 11.

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LITERATURE CITED


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Appendix II

Further evidence for different GST loci:

a preliminary study of the primary protein structure
INTRODUCTION

Since Ingram (1958) first characterized abnormal haemoglobins by "fingerprinting" or peptide mapping, this technique has been shown to be a useful tool for the identification of amino acid sequence differences among closely related molecules. Recent advances now permit peptide mapping at the 1 nmol level (Whittaker and Moss, 1981). Furthermore, peptide mapping is a standard procedure used in the determination of the total amino acid sequence of a protein.

Human glutathione S-transferases have been shown to consist of various isozymes with different characteristics. Dao et al. (1984) reported the N-terminal sequence analysis and amino acid composition of a glutathione S-transferase isozyme purified from human placenta. Subsequently, Alin et al. (1985), recently, described the N-terminal sequences and amino acid compositions of three human glutathione S-transferase isozymes including the acidic (π), the near-neutral (µ), and the basic (α-ε) forms. The acidic (π) isozyme was purified from placenta and the near-neutral (µ) and basic (α-ε) isozymes were purified from liver. The results for the acidic (π) isozyme reported by Alin et al. (1985) are in full agreement with those for a placental glutathione S-transferase isozyme reported by Dao et al. (1984). Furthermore, these results clearly showed different N-terminal amino acid sequences between three glutathione S-transferase isozymes.
The present experiments were designed to:

1) compare amino acid compositions and peptide maps of three isozymes, GST-1, GST-2 and GST-3, which were purified from liver and lung, in order to further investigate their relationships.

2) compare amino acid compositions and N-terminal amino acid sequences of these isozymes with the acidic (π), the near-neutral (μ), and the basic (α-ε) isozymes, which were purified from placenta and liver by other research groups.

Unfortunately, due to technical difficulties and a shortage of time, these studies could not be successfully completed. However, some of the data obtained can be considered with certain reservations.

MATERIALS AND METHODS

Amino acid composition was determined by the standard methods according to Spackman et al. (1958). The amino acids were analyzed on a Beckman Model 119CL amino acid analyzer with a Beckmann 126 Data System.

Peptide mapping was carried out on thin layer chromatography plates (Macherey-Nagel Polygram Cell 300, Düren, West Germany) by the method of Whittaker and Moss (1981). Peptides were detected after development with fluorescamine (0.001% in acetone) ('Fluram', Roche, Switzerland) and then ninhydrin (0.05% in the mixture of 1 ml of pyridine, 1 ml of acetic acid and 200 ml of acetone). The amino-terminal
sequence analysis was carried out on a Beckman 890M protein sequencer.

RESULTS AND DISCUSSION

Tables AP-1, 2 and 3 show the amino acid compositions of GST-1, GST-2 and GST-3. Since the studies reported in previous chapters indicate that these isozymes are probably equivalent to glutathione S-transferases µ, α-ε, and π respectively, these results are compared with those of Alin et al. (1985).

The purified samples of GST-1 and GST-3 were found to be contaminated with glutathione which surprisingly was not eliminated from the preparations by dialysis, additional ion exchange chromatography, or additional gel filtration. This contaminant is believed to be derived from the breakdown products of the glutathione-agarose affinity matrix utilized in the purification of GST-1 and GST-3. The GST-2 used in these experiments contained all the cathodal isozymes and was not finally purified on the glutathione-agarose affinity matrix. The contamination of these samples resulted in the detection of large amounts of glutamate, glycine and cystine in all analyses, and has, therefore, precluded the determination of complete amino acid compositions, peptide maps and N-terminal sequences of GST-1 and GST-3.

Because of the contamination, the amino acid compositions of GST-1 and GST-3 could not be calculated directly
Appendix II. Structural properties

Table AP-1. Amino acid composition of GST-1

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>GST-1</th>
<th>Near-neutral (µ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp (D)</td>
<td>24.2</td>
<td>25.3</td>
</tr>
<tr>
<td>Thr (T)</td>
<td>7.2</td>
<td>27.2</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>16.7</td>
<td>22.2</td>
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<td>Glu (E)</td>
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<td>Gly (G)</td>
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</tr>
<tr>
<td>Ala (A)</td>
<td>15.2</td>
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</tr>
<tr>
<td>Cys (C)</td>
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<td>9.7</td>
</tr>
<tr>
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<tr>
<td>Met (M)</td>
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</tr>
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</tr>
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<td>Tyr (Y)</td>
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</tr>
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<td>Phe (F)</td>
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<td>12.2</td>
</tr>
<tr>
<td>His (H)</td>
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<tr>
<td>Lys (K)</td>
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<td>12.2</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>107.4</td>
<td>108.5</td>
</tr>
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</table>

Number of residue (NR) 256.0  242.3
Subunit M.W. (MW) 27,500  26,300

MW/NR 107.4  108.5

a: The data in parentheses are derived from the results of the near-neutral (µ) isozyme.
b: the data derived from Alin et al. (1985).
c: Thr/Ser not resolved.
d: Integration affected by base-line change.
Table AP-2. Amino acid composition of GST-2

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>GST-2</th>
<th>Basic (a-ε) a</th>
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<tbody>
<tr>
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<td>Leu (L)</td>
<td>34.4</td>
<td>26.3</td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>11.2</td>
<td>9.4</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>11.0</td>
<td>9.1</td>
</tr>
<tr>
<td>His (H)</td>
<td>3.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Lys (K)</td>
<td>24.8</td>
<td>20.3</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>10.2</td>
<td>10.6</td>
</tr>
</tbody>
</table>

Number of residue (NR) 244.5 218.6

Subunit M.W. (MW) 26,900 23,500

MW/NR 110.0 107.5

a: the data derived from Alin et al. (1985).
b: Thr/Ser not resolved.
### Table AP-3. Amino acid composition of GST-3

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>GST-3 a</th>
<th>Acidic (π) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp (D)</td>
<td>28.6</td>
<td>22.1</td>
</tr>
<tr>
<td>Thr (T)</td>
<td>10.3</td>
<td>9.5</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>23.1</td>
<td>13.5</td>
</tr>
<tr>
<td>Glu (E)</td>
<td>(25.7)</td>
<td>25.7</td>
</tr>
<tr>
<td>Pro (P)</td>
<td>14.8</td>
<td>13.4</td>
</tr>
<tr>
<td>Gly (G)</td>
<td>(21.5)</td>
<td>21.5</td>
</tr>
<tr>
<td>Ala (A)</td>
<td>16.4</td>
<td>16.6</td>
</tr>
<tr>
<td>Cys (C)</td>
<td>(4.0)</td>
<td>4.0</td>
</tr>
<tr>
<td>Val (V)</td>
<td>9.8</td>
<td>13.9</td>
</tr>
<tr>
<td>Met (M)</td>
<td>2.0</td>
<td>3.3</td>
</tr>
<tr>
<td>Ile (I)</td>
<td>6.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Leu (L)</td>
<td>22.2</td>
<td>32.4</td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>14.9</td>
<td>12.2</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>6.7</td>
<td>7.4</td>
</tr>
<tr>
<td>His (H)</td>
<td>4.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Lys (K)</td>
<td>16.1</td>
<td>13.0</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>5.6</td>
<td>8.3</td>
</tr>
</tbody>
</table>

**Number of residue (NR)** 232.4 226.6

**Subunit M.W. (MW)** 25,100 25,000

**MW/NR** 108.0 110.3

* a: The data in parentheses are derived from the results of the acidic (π) isozyme.
  b: the data derived from Ålin et al. (1985).
Appendix II. Structural properties

and some calculations have been made after the determination of a calibration factor from the data by Alin et al. (1985). Leu was chosen as a standard and the calculations carried out as follows: Alin et al. (1985) reported 26.5 residues of Leu in the amino acid composition of the near-neutral (µ) isozyme. In the present data for GST-1, the Leu concentration was 3.168 nmoles. Therefore a calibration factor was determined, 3.168 nmoles/26.4 residues = 0.12 nmoles/residue. Finally each amino acid concentration was divided by the calibration factor (0.12) to obtain residue numbers for each amino acid. Similar calibrations were made for GST-3.

The peaks for Pro and Ala from GST-1 could not be determined in the present experiments, because of the interference of additional unidentified chromatographic peaks. In addition the proportion of Ser in the data obtained for GST-1 and GST-3 is relatively higher than that reported in Alin et al. (1985) for isozymes µ and π. This high value may also result from interference by the high glutathione-agarose content in the GST-1 and GST-3 samples.

Despite calibration adjustments to be data for GST-1 and GST-3, the amino acid compositions of GST-1, GST-2 and GST-3 appear to be largely similar to the results obtained by Alin et al. (1985) for the µ, α-ε, and π isozymes. GST-1 and GST-2 were purified from liver and Alin et al. (1985) also purified the near-neutral (µ) and basic (α-ε) isozymes from liver. On the other hand, GST-3 was purified from
lung in the present studies and the acidic isozyme (\(\pi\)) studied by Ålin et al. (1985) was purified from placenta. The similarities of these two isozymes strongly support the proposal of the present study, that GST-3 and the acidic isozyme purified from different tissues by others are identical.

Figure AP-1 shows a peptide map of a trypsin digest of GST-2. A large number of clearly defined peptides were apparent after ninhydrin staining. Before ninhydrin staining several peptides could be detected under UV light and Fluram staining. Because of the contamination of glutathione-agarose matrix, peptide maps for GST-1 and GST-3 were not successfully obtained for comparison.

Attempts were also made to obtain amino terminal sequence data to compare the different isozymes. Because it was largely free of contamination, a sample of GST-2 was subjected to automated N-terminal sequence analysis. No sequence was obtained, indicating that this GST-2 preparation was blocked at the N-terminal. This finding can be compared with the recent report of Ålin et al. (1985) who also reported that the N-terminal of the basic (\(\alpha-\epsilon\)) isozymes they studied was also blocked. This finding therefore also supports the earlier conclusion that the cathodal GST-2 isozymes are equivalent to the basic \(\alpha-\epsilon\) isozymes purified by others. In addition, since the preparation of GST-2 used in the studies contained both GST-2*1 and GST-2*2, it is evident that both isozymes must be blocked at the N-terminal.
Figure AP-1. Peptide map of GST-2

The lyophilized sample (2nM) was treated with performic acid to cleave disulfide bonds for 2-4 hours on ice. The denatured protein was treated by trypsin with TPCK (224 U/mg, 84% protein, Worthington Biochemical Corp., Freehold, N.J., USA) at 37°C for 4 hours. The concentration of trypsin was approximately 2% of the sample weight.

A combination of chromatography and electrophoresis was performed for the separation of peptides. First, ascending thin-layer chromatography was carried out in BAWP (n-butanol:glacial acetic acid:pyridine:double distilled water = 15:3:12:10) for 30 minutes. Secondly, electrophoresis was performed with running buffer (50 ml of pyridine and 50 ml of glacial acetic acid in 2 litres, pH4.7) at 1000 V constant (55-60 mA) for 20 minutes at room temperature. Finally, ascending chromatography was carried out with BAWP for 5 hours.

For the localization of peptides, a freshly made solution of ninhydrin solution (0.05% in a mixture of 1 ml of pyridine, 1 ml of acetic acid and 200 ml of acetone) was sprayed on the sheet.
Electrophoresis at 1000 V for 20 min.
REFERENCES


