# GLUTATHIONE S-TRANSFERASES: CATALYTIC AND MOLECULAR PROPERTIES OF MU-CLASS AND THETA-CLASS ISOENZYMES

BY

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## DECLARATION OF ORIGINALITY

I declare that the work presented herein and the composition of this thesis is my own.

Amanda J. Hussey

# CONTENTS

**Abstract**

The cytosolic Mu-class glutathione S-transferases (GST) present in human liver, skeletal muscle and testis comprise three distinct neutral-type subunits ($N_1$, $N_2$ and $N_3$) which hybridize to form homodimers or heterodimers. The two $N_1$-type subunits, which represent allelic variants encoded by the hepatic Mu-class GST locus, have been designated $N_1^a$ and $N_1^b$. Three isoenzymes containing $N_1$-type subunits have been purified from one liver specimen by sequential affinity chromatography, hydroxyapatite chromatography and chromatofocusing. The skeletal muscle GST $N_1^aN_2$, $N_1^bN_2$, $N_2N_2$ and $N_2N_3$ were purified by a combination of affinity chromatography and anion-exchange f.p.l.c. followed by either chromatofocusing f.p.l.c. or hydroxyapatite h.p.l.c. In muscle the expression of the $N_1$-type subunit, but not of the $N_2$ and $N_3$ subunits, was found to differ from specimen to specimen. The $N_1$-type subunits were absent from about 50% of samples analyzed, and the purification results from four different specimens is presented to illustrate the phenotypic variation of skeletal muscle GST. The isolation of a homodimeric Mu-class isoenzyme, called GST $N_3N_3$, from human testicular tissue was achieved in two chromatographic steps; namely affinity chromatography followed by anion-exchange f.p.l.c.

The neutral-type GST subunits have been defined by the decreasing isoelectric points of the homodimeric enzymes; GST $N_1^aN_1^a$, $N_1^bN_1^b$, $N_2N_2$ and $N_3N_3$ have pI values of 6.1, 5.5, 5.3 and 5.0. SDS/PAGE showed that $N_1$, $N_2$ and $N_3$ have $M_r$ values of 26700, 26000 and 26300 respectively. The $N_1$, $N_2$ and $N_3$ subunits are catalytically distinct, with the $N_1$-type subunits possessing high activity for *trans*-4-phenyl-3-buten-2-one and $N_2$ having high activity towards 1,2-dichloro-4-nitrobenzene. The N-type subunits may also be distinguished immunochemically; antisera raised against the testicular GST $N_3N_3$ cross-reacted with the $N_3$ subunit, and showed no reactivity towards either the $N_1$-type or the $N_2$ subunit. N-terminal amino acid sequence analysis supported the electrophoretic evidence that the $N_2$ subunit in GST $N_1^aN_2$, $N_1^bN_2$, $N_2N_2$ and $N_2N_3$ represents the same polypeptide. The peptides obtained from CNBr digests of $N_2$ were subjected separately to automated amino acid sequencing, and the results indicate that $N_2$ is distinct but closely related to the protein encoded by the human Mu-class cDNA clone $GTH_4$ [DeJong, Chang, Whang-Peng, Knutsen & Tu (1988) Nucleic Acids Res. **16**, 8541-8554]. GST $N_2N_2$ is probably identical with GST4 [Board, Suzuki & Shaw (1988) Biochim. Biophys. Acta **953**, 214-217], as over the 24 N-terminal residues of GST4 there is complete identity between the two enzymes. The $N_3$ subunit, which is expressed in skeletal muscle and testis possesses a blocked N-terminus. Automated amino acid sequencing of a CNBr-derived peptide

allowed 14 residues of the $N_3$ subunit to be identified. This data indicated that testicular GST $N_3N_3$ is likely to be identical to brain/testis $\mu$ [Campbell, Takahashi, Abramovitz, Peretz & Listowsky (1990) J Biol. Chem. **265**, 9188-9195].

Purification schemes are described for GST's from human and murine liver that catalyse the conjugation of 1-menaphthyl sulphate (1-MS) with reduced glutathione. The human enzyme which metabolises 1-MS is a homodimer comprising subunits of $M_r$ 25100. Immunochemical experiments have shown that this enzyme and related murine forms are members of the class Theta GST. Automated amino acid sequence analysis of the human isoenzyme have confirmed the immunochemical data and shown that it is related to human GST $\theta$ described previously [Meyer, Coles, Pemble, Gilmore, Fraser & Ketterer (1991) Biochem. J. **274**, 409-414]. However, the human enzyme which catalyses the conjugation of 1-MS with glutathione is distinct from GST $\theta$ and has been designated GST $T_2T_2$. The various data which indicate GST $\theta$ and GST $T_2T_2$ represent distinct isoenzymes are discussed.

**Figures and Tables**

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**Abbreviations**

| | |
|---|---|
| BCNU | 1,3-bis(2-chloroethyl)-1-nitrosourea |
| BP | benzo(a)pyrene |
| BPDE | benzo(a)pyrene-7,8-diol-9,10-epoxide |
| BSP | bromosulphophthalein |
| CDNB | 1-chloro-2,4-dinitrobenzene |
| CNBr | cyanogen bromide |
| CuOOH | cumene hydroperoxide |
| DCNB | 1,2-dichloro-4-nitrobenzene |
| EA | ethacrynic acid |
| EDTA | ethylenediaminetetra-acetic acid |
| EH | epoxide hydrolase |
| EPNP | 1,2-epoxy-3-(*p*-nitrophenoxy)propane |
| f.p.l.c. | fast protein liquid chromatography |
| FAEE | fatty acid ethyl ester |
| GSH | reduced glutathione |
| GSSG | glutathione disulphide |
| GST | glutathione S-transferase |
| h.p.l.c. | high pressure liquid chromatography |
| 4-HDE | 4-hydroxydecinal |
| 5-HCR | 5-hydroxymethylchrysene |
| 4-HNE | 4-hydroxynonenal |
| IEF | isoelectric focusing |
| 1-MS | 1-menaphthyl sulphate |
| MFO | mixed function oxygenase |
| *p*-NBC | *para*-nitrobenzyl chloride |
| PTH | phenylthiohydantoin |
| PAH | polycyclic aromatic hydrocarbon |
| PG | prostaglandin |
| SDS/PAGE | sodium dodecyl sulphate/polyacrylamide-gel electrophoresis |
| *t*-PBO | *trans*-4-phenyl-3-buten-2-one |

**Amino acids: single letter code**

| | |
|---|---|
| A | alanine |
| C | cysteine |
| D | aspartic acid |
| E | glutamic acid |
| F | phenylalanine |
| G | glycine |
| H | histidine |
| I | isoleucine |
| K | lysine |
| L | leucine |
| M | methionine |
| N | asparagine |
| P | proline |
| Q | glutamine |
| R | arginine |
| S | serine |
| T | threonine |
| V | valine |
| W | tryptophan |
| Y | tyrosine |

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# PART I

# INTRODUCTION

# Chapter 1 Enzymology of the glutathione S-transferases

## 1.01 General introduction

The glutathione S-transferases (GST) are a complex group of detoxification enzymes that catalyse the conjugation of glutathione to various xenobiotic and endogenous electrophiles (Mannervik and Danielson, 1988; Ketterer, 1988). This reaction is the first step in the formation of mercapturic acids, a pathway that is important as a means of rendering these hydrophobic compounds water soluble and thereby aiding their elimination from the body (Smith *et al.*, 1977; Chasseaud, 1979). In addition to their conjugation activity, some of these enzymes bind certain non-substrate ligands and this has led to the suggestion that they may also serve a function as intracellular carrier proteins (Litwack *et al.*, 1971; Listowsky *et al.*, 1988). These multifunctional proteins are widely distributed in both the animal and the plant kingdom and in all species are represented by a number of isoenzymes.

The mammalian GST, which are present as soluble and membrane bound forms, can be subdivided into classes which share structural and functional homology. Three multigene families, commonly referred to as Alpha, Mu and Pi, encode the major cytosolic forms of GST (Alin *et al.*, 1985a, Mannervik *et al.*, 1985, Hayes and Mantle, 1986a). Recently, however, soluble isoenzymes which do not belong to these three classes have been isolated, and a fourth class of GST, designated Theta, has been recognised (Hiratsuka *et al.*, 1990; Meyer *et al.*, 1991). Microsomal GST, which are unrelated to the cytosolic forms, have also been purified from various sources (Morgenstern *et al.*, 1982, 1985; McLellan *et al.*, 1989).

The GST isoenzymes of the rat have been the most extensively studied. The initial investigations into the structure, function and tissue distribution of the rat GST have provided a foundation for subsequent research into the expression of these enzymes in humans and other species.

## 1.02 Historical background

GST activity was first identified in rat liver extracts, in the early 1960's, by two independent groups of workers. These groups reported that hepatic fractions catalysed the conjugation of glutathione with 1,2-dichloro-4-nitrobenzene (Booth *et al.*, 1961) and bromosulphophthalein (Combes and Stakelum, 1961). Subsequent reports demonstrated the existence of several different types of GST activity in rat liver and initially these enzymes were described according to the type of reaction they catalysed. The GST recognised by Booth *et al.* (1961), which was active with halogenated aromatic hydrocarbons was, therefore, named glutathione S-aryltransferase (Grover and Sims, 1964). During the first five years following their discovery, many other GST substrates were reported and other types of GST-catalysed reactions identified: glutathione S-epoxidetransferase, catalysing the reaction of glutathione with many epoxides; glutathione S-alkyltransferase, which catalysed the S-alkylation of various alkylhalides; glutathione S-aralkyltransferase, which utilised aralkylhalides and esters as substrates; and glutathione S-alkenetransferase which catalysed the reaction of glutathione with $\alpha\beta$-unsaturated compounds (Boyland and Chasseaud, 1969).

Later reports, which described the purification of some of these rat liver GST to homogeneity, confirmed that multiple forms existed, however, they also demonstrated that the individual isoenzymes had broad and overlapping substrate specificities (Fjellstedt *et al.*, 1973; Pabst *et al.*, 1973; Habig *et al.*, 1974a; Habig *et al.*, 1976). Therefore, rather than using a classification based upon the structure of the substrate, these purified enzymes were named according to their chromatographic properties. Jakoby and co-workers identified six rat liver GST which were termed AA, A, B, C, D and E in reverse order of their elution from CM-cellulose columns (Habig *et al.*, 1976). Glutathione S-epoxidetransferase, later referred to as transferase E, was the first of these enzymes to be purified from rat liver (Fjellstedt *et al.*, 1973). In the same year Gillham (1973) partially purified an additional GST from a rat hepatic fraction that was active towards the substrate 1-menaphthyl sulphate and was later designated transferase M.

An organic-anion-binding protein called ligandin was also purified at this time (Litwack *et al.*, 1971). Subsequent reports demonstrated that ligandin possessed glutathione conjugating activity towards bromosulphophthalein (Kaplowitz *et al.*, 1973) and provided evidence that it was identical to GST B (Habig *et al.*, 1974b). However, it was later shown

that the different preparations of ligandin obtained from various laboratories contained different or heterogeneous GST mixtures (Hayes *et al.*, 1979; Hayes and Clarkson, 1982).

Bass *et al.*, (1977) used SDS/polyacrylamide-gel electrophoresis to resolve the three major subunits which comprise the cytosolic GST of rat liver. These polypeptides which were isolated as a bromosulphophthalein-binding Y-fraction were described as Ya, Yb and Yc; a classification which provides a basis for one of the currently used systems of rat GST nomenclature (see section 2.01b).

**1.03 Glutathione**

1.03a Structure, function and metabolism of glutathione

Glutathione, a characteristic and essential component of all eukaryotic cells, is a tripeptide with the sequence γglutamic acid-cysteine-glycine (Figure 1.01). This small molecule, the most abundant low-molecular-mass thiol in nature, has an involvement in many biological processes, including protein synthesis, amino acid transport and enzyme activity (Kowsner and Kowsner, 1976; Meister and Anderson, 1983). Glutathione is also a powerful antioxidant which affords the cell protection against the toxic products of oxygen metabolism (Mannervik, 1986; Deneke and Fanburg, 1989).

Within mammalian cells a critical balance is maintained between the readily interconvertible reduced (GSH) and oxidised (GSSG) forms of glutathione. High intracellular levels of GSH are generally found with concentrations which vary between 0.5-10.0 mM depending on the tissue, and the growth, nutritional state and hormonal balance of the organism (Meister and Anderson, 1983). Whereas, significantly lower concentrations of glutathione disulphide (4-50 μM) are essential for protein synthesis to occur and the action of certain enzymes (Kowsner, 1976).

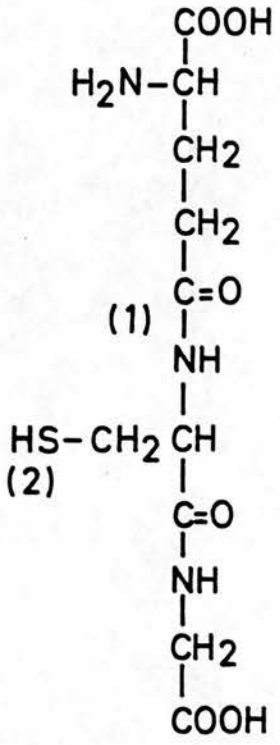
Intracellular GSH levels are dependant on the balance between the rate of its utilization, in terms of oxidation, conjugation and degradation, and the rates of its synthesis and reduction of GSSG. The oxidation of GSH and the reduction of GSSG are mediated by the actions of glutathione peroxidase and glutathione reductase respectively. Glutathione is synthesised intracellularly, from its constituent amino acids, by a two step ATP requiring process. The first reaction involves the formation of the γglutamyl peptide link with cysteine, catalysed by γglutamylcysteine synthetase and this is followed by the formation

**Figure 1.01 The structure of glutathione**

The structure of reduced glutathione (GSH), $\gamma$glutamylcysteinylglycine.

(1) The glutamic acid residue is joined to the cysteine residue with an unusual peptide linkage which involves the $\gamma$-carboxyl group rather than the $\alpha$-carboxyl group.

(2) The cysteinyl thiol group.




GLUTATHIONE

**Figure 1.01**

of a peptide bond between the cysteine moiety of γglutamylcysteine and glycine, catalysed by glutathione synthetase. The breakdown of GSH, on the other hand, occurs extracelluarly and is catalysed by γglutamyl transpeptidase. This membrane bound enzyme transfers the γglutamyl moiety from GSH to an amino acid. The resulting γglutamyl amino acid and cysteinylglycine may then be transported into the cell where they become available as substrates for glutathione synthesis (Meister and Anderson, 1983).

Glutathione possesses a number of hydrophillic functional groups and is highly water soluble. The nucleophilic thiol group, however, is the most important chemically reactive group allowing GSH to react readily with compounds containing an electrophilic carbon, nitrogen, sulphur or oxygen atom. These reactions are generally catalysed by glutathione S-transferases, however in many instances they may also proceed non-enzymatically (Chasseaud,1979). The conjugation of glutathione with electrophilic xenobiotics generally results in the formation of less toxic compounds prior to their elimination from the body. Recently, a glutathione S-conjugate efflux pump, which actively transports both foreign and endogenous compounds out of cells, has been identified (Ishikawa, 1989; Ishikawa *et al.*, 1989). The glutathione S-conjugate may be excreted directly into bile or, following conversion to an N-acetyl cysteine conjugate (or mercapturic acid), may be excreted into urine (Chasseaud,1979).

### 1.03b Mercapturic acid biosynthesis

Mercapturic acids are the classical excretion products of xenobiotics (Boyland and Chasseaud,1969; Mannervik, 1985). These compounds are the end product of a series of reactions which take place in the liver and kidneys of most species and involve the stepwise degradation of the glutathione moiety, of the GSH-electrophile conjugate, followed by acetylation.

The first step in this detoxification pathway, depicted in Figure 1.02, is the conjugation of glutathione with electrophilic compounds catalysed by the glutathione S-transferases. In a reaction catalysed by γglutamyl transpeptidase, the membrane bound enzyme which is also involved in GSH degradation (see section 1.03a), the γglutamyl moiety of the GSH conjugate may then be removed and transferred to amino acid acceptors. The resulting cysteinylglycine conjugate is then hydrolysed by dipeptidase to form an S-cysteine

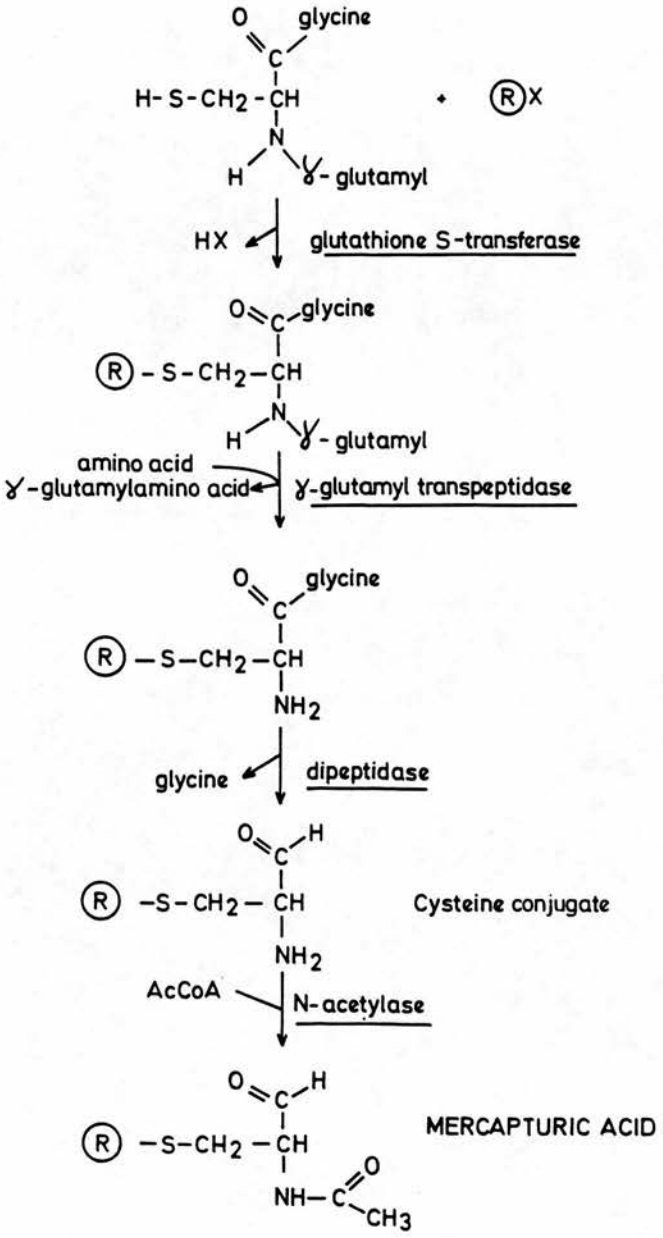



**Figure 1.02 The mercapturic acid pathway**

derivative and glycine. The final stage in the formation of the mercapturic acid is the N-acetylation of the cysteine conjugate catalysed by an acetylase.

An alternative metabolic pathway for the biotransformation of the cysteine conjugate involves the cleavage of the cysteine moiety by $\beta$-lyase resulting in the elimination of pyruvate and ammonia and the formation of a mercaptan. The thiol group of the mercaptan may be blocked by either glucuronydation or methylation and the resulting glucuronide or methylthio derivative can be excreted (Pickett and Lu, 1989).

### 1.04 Model substrates

The cytosolic GST are dimeric proteins which posses two catalytically independent subunits (Danielson and Mannervik, 1985). Each subunit contains an active centre composed of two binding sites; one with a high affinity for glutathione (G-site) and a second hydrophobic site for the electrophilic substrate (H-site) (Jakobson *et al.*, 1977; Mannervik, 1985). It is thought that GST act as catalysts by bringing the two substrates into close juxtaposition (the "proximity effect") and promoting the ionization of GSH by lowering the $pK_a$ of the thiol moiety (Jakoby, 1978).

Whereas, the requirement of the glutathione S-transferases for GSH is highly specific, with only homoglutathione ($\gamma$glutamylcysteinylalanine) and $\gamma$glutamylcysteine serving as possible alternatives (Habig *et al.*, 1974a; Sugimoto *et al.*, 1985), the requirements for the second substrate, hydrophobicity and a sufficiently electrophilic centre, are such that an extremely wide variety of compounds may serve as substrates for these enzymes. Most of the compounds which were originally used by researchers for the analysis of GST enzymic activity were products of the chemical industry chosen because their conjugation with GSH results in an optical density change which could be followed spectro-photometricaly. The compound which is often considered as the "universal substrate" for the GST is 1-chloro-2,4-dinitrobenzene (CDNB). It is apparent, however, that certain GST forms express either low or undetectable activity towards this compound (Meyer *et al.*, 1984, 1991; Hiratsuka *et al.*, 1990), and as a consequence there is a danger that new forms may be overlooked unless a suitable substrate is chosen.

1.04a Types of reaction catalysed by GST

The nucleophilic attack of the glutathione thiolate ion ($GS^-$) may be directed against sufficiently electrophilic carbon, oxygen, sulphur and nitrogen atoms, thereby producing a variety of reaction types. These reactions include not only those which result in glutathione conjugation but also reactions which involve hydroperoxide reduction and isomerization.

The conjugation reactions, with a resultant thioether product, involve either substitution or addition reactions. The nucleophilic attack of $GS^-$ on the aromatic carbon of 1-chloro-2,4-dinitrobenzene, results in the nucleophilic displacement of a chloro substituent, and is an example of the former type of reaction. The conjugation of glutathione with the $\alpha\beta$-unsaturated carbonyl compound ethacrynic acid, on the other hand, is an example of an addition reaction. In this case the glutathione reacts additively with the $\beta$-carbon atom of an activated $\alpha\beta$ double bond (Boyland and Chasseaud, 1969). These two reactions are depicted in Figure 1.03. Various epoxides also undergo addition reactions in which the nucleophilic attack of glutathione results in the opening of an oxirane ring (Fjellstedt *et al.*, 1973).

GST-mediated hydroperoxide reduction involves a nucleophilic attack at an electrophilic oxygen. This reaction is believed to occur in two stages involving the enzymatic formation of an unstable sulphenic acid intermediate (GSOH), followed by the non-enzymatic nucleophilic attack of a second molecule of GSH to form glutathione disulphide (GSSG) and water (Prohaska *et al.*, 1980). The conversion of the GST substrate cumene hydroperoxide to the corresponding cumyl alcohol is shown in Figure 1.03. GSH may be regenerated from GSSG by the action of glutathione reductase:

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

The GST catalysed reduction of hydroperoxides represents selenium-independent glutathione peroxidase activity. The enzymes responsible for this activity will only act upon organic hydroperoxides, unlike selenium-dependant glutathione peroxidase which also utilizes $H_2O_2$ as a substrate (Laurence and Burk, 1976).

**Figure 1.03 Examples of GST catalysed reactions**

Panels a, b, c and d depict various types of reaction catalysed by GST:

a) The conjugation of glutathione to 1-chloro-2,4-dinitrobenzene involving a substitution reaction at an electrophilic carbon atom.

b) The conjugation of glutathione to ethacrynic acid involving an addition reaction at an electrophilic carbon.

c) Glutathione-mediated reduction of cumene hydroperoxide following an attack at an electrophilic oxygen.

d) Glutathione-mediated isomerization of $\Delta^5$ androstene-3,17-dione following an attack at an electrophilic carbon.

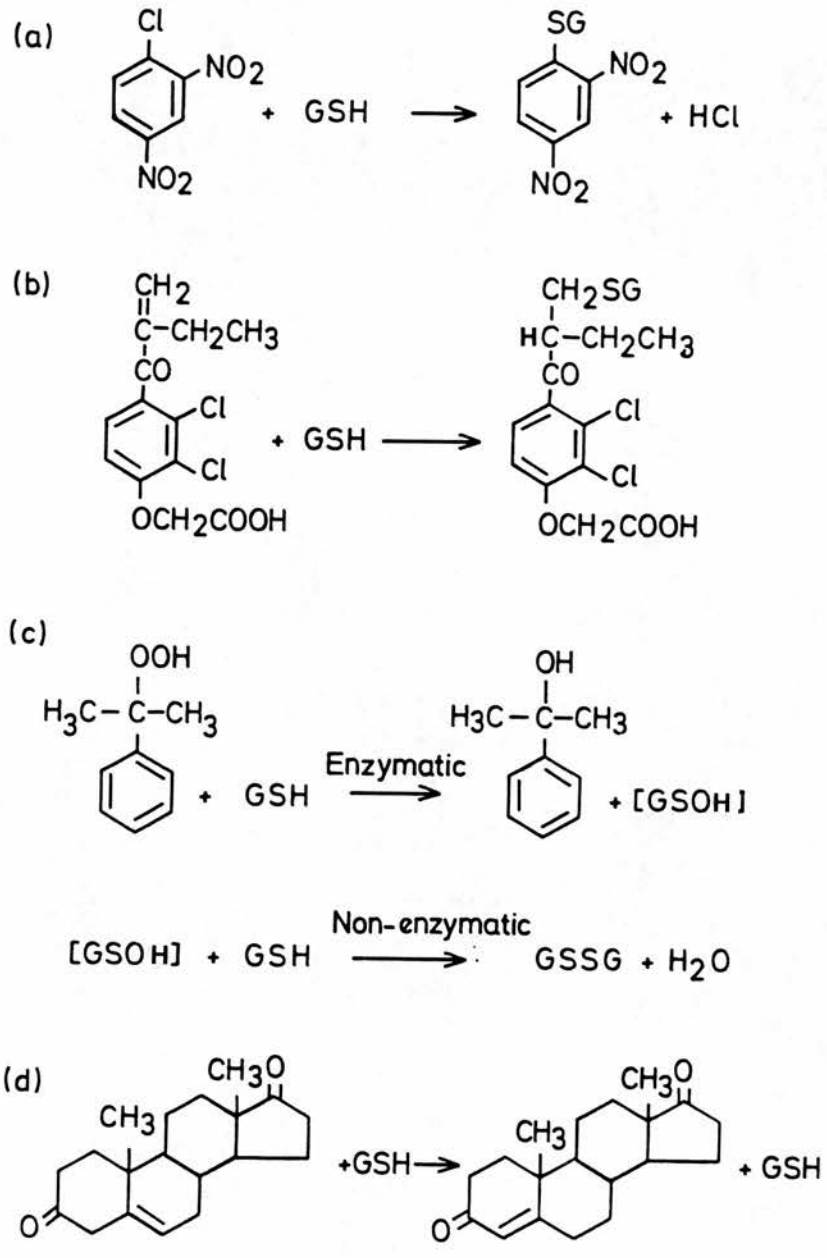

(a)
Cl
NO2
NO2
+ GSH
SG
NO2
NO2
+ HCl
(b)
CH2
C–CH2CH3
CO
Cl
Cl
OCH2COOH
+ GSH
CH2SG
HC–CH2CH3
CO
Cl
Cl
OCH2COOH
(c)
OOH
H3C–C–CH3
+ GSH
Enzymatic
OH
H3C–C–CH3
+ [GSOH]
[GSOH] + GSH
Non-enzymatic
GSSG + H2O
(d)
CH3
CH3O
O
+GSH
CH3
CH3O
O
+ GSH

**Figure 1.03**

Certain GST also catalyse the isomerization of a double bond system as in the conversion of $\Delta^5$ androstene-3,17-dione to $\Delta^4$ androstene-3,17-dione (Figure 1.03). In this type of reaction GSH acts as a co-enzyme and is not consumed; it is thought that an unstable GSH adduct is formed which, upon decomposition, releases GSH and the more stable isomer (Habig, 1983). Compounds which contain electrophilic nitrogen (eg. organic nitrate esters) and sulphur atoms (eg. organic thiocyanates and disulphides) are also targets for GST catalysed reactions (Keen *et al.*, 1976).

1.04b Characterization of enzymes using different substrates

Individual GST forms have distinct catalytic properties and those enzymes which are structurally related often posses characteristically high specific activities with particular compounds (Mannervik *et al.*, 1985). For example, both rat and human Alpha-class GST demonstrate high peroxidase activity towards cumene hydroperoxide (Hayes *et al.*, 1987a; Stockman *et al.*, 1987). Steroid isomerase activity, with the model substrate $\Delta^5$ androstene-3,17-dione, is also a characteristic of certain rat and human Alpha-class GST (Benson *et al.*, 1977; Hayes *et al.*, 1987a). In general, Mu-class enzymes have been found to be the most active of the GST towards epoxides (Mannervik *et al.*, 1985). Styrene oxide, a metabolite of the chemical industry product styrene, has been shown to be a good substrate for human hepatic Mu-class GST (Warholm *et al.*, 1983). Pi-class GST, on the other hand, are distinguished by their relatively high activity towards ethacrynic acid (Mannervik *et al.*, 1985; Stockman *et al.*, 1987). Compounds used recently for the identification of Theta-class GST include 1-menaphthyl sulphate (Hiratsuka *et al.*, 1990) and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (Meyer *et al.*, 1984, 1991).

Information on substrate specificities may also be useful in distinguishing between GST isoenzyme forms within a class. Substrates which have been found to discriminate between individual members of the rat Mu-class GST include *trans*-4-phenyl-3-buten-2-one, 1,2-dichloro-4-nitrobenzene and bromosulphophthalein (Hayes, 1986). The structures of the compounds which are characteristic for Mu- and Theta-class GST are shown in Figure 1.04.

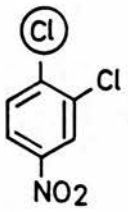
**Figure 1.04 Characteristic substrates for Mu- and Theta-class GST**

Mu-class GST substrates:
a) 1,2-Dichloro-4-nitrobenzene
b) *trans*-4-Phenyl-3-buten-2-one
c) Bromosulphophthalein

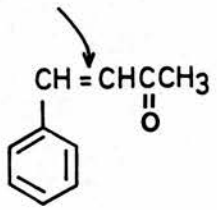
Theta-class GST substrates:
d) 1-Menaphthyl sulphate
e) 1,2-Epoxy-3-(*p*-nitrophenoxy)propane

Mu

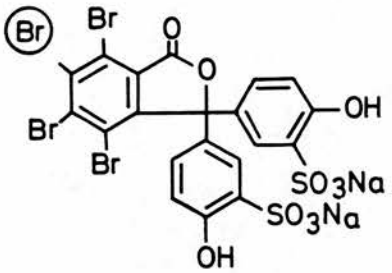
(a)




(b)

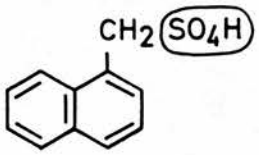



(c)




Theta

(d)




(e)

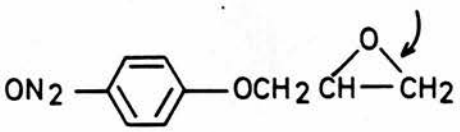



**Figure 1.04**

## 1.05 Xenobiotic substrates

Many xenobiotic electrophiles have the potential to react with cellular macromolecules and thereby cause a variety of toxic effects including mutations and cancer. Complex drug metabolizing enzyme systems exist, within the cell, in order to combat this challenge. These enzymes, which catalyse the activation and detoxification of xenobiotics, are generally described as phase I and phase II. Phase I metabolism includes oxidation, reduction, hydrolysis and hydration reactions, whereas phase II enzymes catalyse various types of conjugation reactions. It is thought that phase I reactions create a reactive functional group on the molecule which may subsequently be attacked by the phase II enzymes (Gibson and Skett, 1986). The microsomal mixed function oxidase (MFO) system performs many different phase I "functionalization" reactions and the reactive intermediates formed by this oxidative metabolism may then be detoxified by conjugation reactions which include glucuronidation, sulphation, methylation and acetylation as well as glutathione conjugation. The xenobiotic compounds which are present in the environment arise as products of the chemical industry, pollutants, natural products and therapeutic drugs.

### 1.05a The detoxification of mutagens and carcinogens by GST

The polycyclic aromatic hydrocarbons (PAH) are common environmental pollutants which have been implicated as aeteological factors in human chemical carcinogenesis (IARC, 1983, 1984). One such PAH which has been studied extensively is benzo(a)pyrene (Gelboin, 1980). The metabolic activation of this compound to various reactive intermediates, including epoxides, phenols and quinones, is performed by the cytochrome-P450-containing mixed function oxidase (MFO) system. Subsequent metabolic steps may involve the hydration of epoxides to dihydrodiols, mediated by the enzyme epoxide hydrolase, which may be followed by further oxygenation of these compounds to form diol epoxides.

Several of the reactive intermediates arising during the metabolism of benzo(a)pyrene have been shown to be Mu-class GST substrates (Figure 1.05). A human hepatic Mu-class enzyme , identified first in 1980 (Warholm *et al.*, 1980), was subsequently shown to have a high specific activity, in comparison with Alpha- and Pi-class enzymes, towards benzo(a)pyrene 4,5-oxide (Warholm *et al.*, 1981, 1983). This compound and other K-region epoxides yield positive results in bacterial mutagenicity tests although they have

**Figure 1.05 Metabolism of benzo(a)pyrene**

The metabolic activation of the polycyclic aromatic hydrocarbon benzo(a)pyrene (BP) may involve the formation of the mutagenic compounds BP-4,5-oxide and/or BP-7,8-diol-9,10-epoxide (BPDE). Both of these reactive intermediates have been shown to be substrates for GST enzymes. Microsomal mixed function oxidases (MFO) catalyse the initial epoxidation reactions to form either BP-7,8-oxide or BP-4,5-oxide. Hydration of BP-7,8-epoxide, mediated by epoxide hydrolase (EH), results in the formation of BP-7,8-diol. This compound may then be reactivated by the MFO to form BPDE. GST catalysed conjugations of glutathione to either BP-4,5-oxide or BPDE result in the detoxification of these compounds.

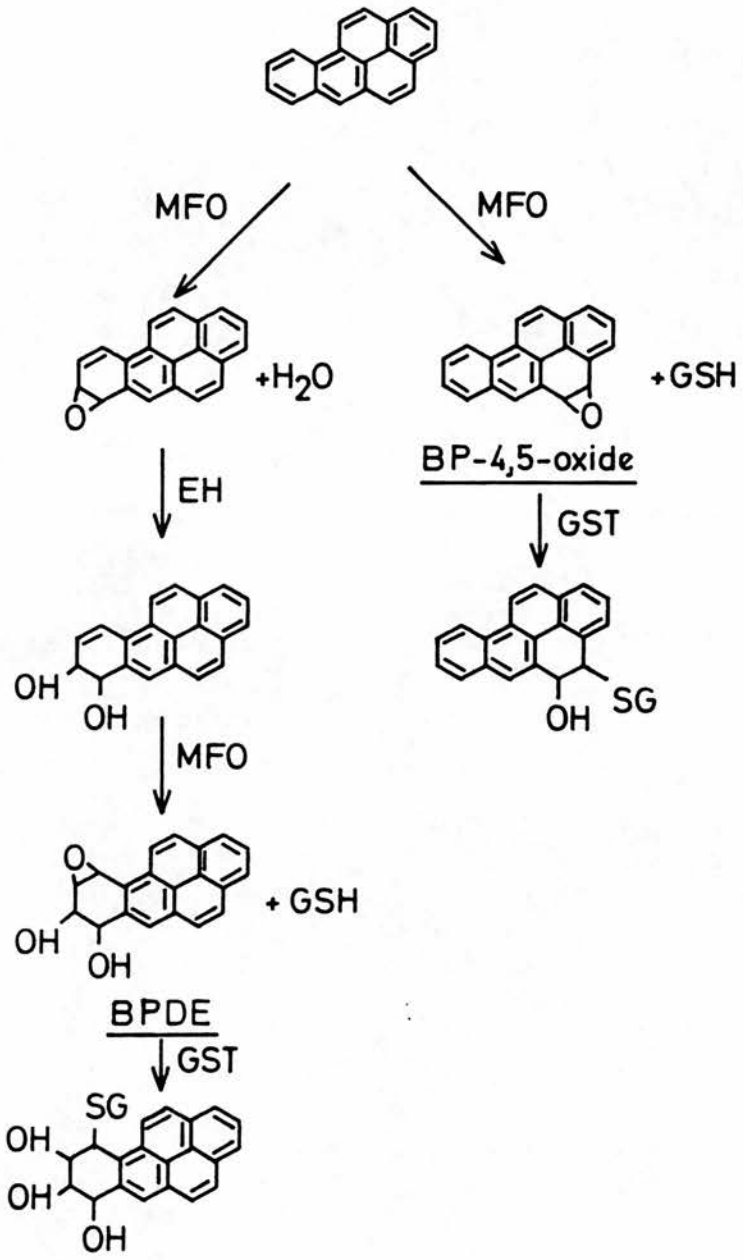

MFO
MFO
+H2O
+GSH
O
O
BP-4,5-oxide
EH
GST
OH
OH
SG
OH
MFO
O
+ GSH
OH
OH
BPDE
GST
SG
OH
OH
OH

**Figure 1.05**

not been directly implicated in carcinogenesis (Coles and Ketterer, 1990). The "Bay region" epoxides, on the other hand, are responsible for the carcinogenic effect of PAH (Jerina and Lehr, 1977). Glutathione conjugation has been shown to be an important protection mechanism against the DNA binding of the "Bay region" diol epoxide benzo(a)pyrene-7,8-diol-9,10-oxide (BPDE) (Hesse *et al.*, 1982). The GST which have high activity towards this metabolite include a rat Mu-class enzyme (Jernstrom *et al.*, 1985), and human Mu- and Pi-class enzymes (Robertson *et al.*, 1986). It is of interest that the nucleophilic attack of GSH on both K-region and "Bay region" epoxides occurs stereoselectively. For instance the conjugation of GSH with BPDE catalysed by rat Mu-class GST $Yb_2Yb_2$ is selective towards the biologically most active (+)-enantiomer (Robertson and Jernstrom, 1986).

Although the formation of epoxide metabolites of PAH's is regarded as a major metabolic pathway yielding carcinogenic compounds the existence of an alternative activation mechanism, involving the formation of reactive sulphate esters, has recently been demonstrated (Watabe *et al.*, 1986; Okuda *et al.*, 1989). The bioactivation of benz(a)anthracene and chrysene, PAH which are regarded as weak carcinogens, involves methylation followed by enzymic oxidation at the methyl carbon, mediated by microsomal monooxygenases, to yield the corresponding hydroxymethylarenes. These potent carcinogens may then be further activated by hydroxysteriod sulphotransferase to the corresponding reactive sulphate esters (Watabe *et al.*, 1987; Okuda *et al.*, 1989). The metabolically formed sulphate esters react with purine bases on both calf thymus and preweanling rat DNA (Watabe *et al.*, 1985; Surh *et al.*, 1987). GSH conjugation, mediated by GST present in rat hepatic cytosol, has been shown to inactivate both 5-hydroxymethyl-chrysene sulphate and 7,12-dihydroxymethylbenz(a)anthracene sulphate and inhibit the formation of DNA adducts (Okuda *et al.*, 1986; Watabe *et al.*, 1987). The metabolic activation and inactivation of 5-methylchrysene, an environmental pollutant found in tobacco smoke, is depicted in Figure 1.06.

Sato and colleagues have recently used GST activity towards 5-hydroxymethylchrysene sulphate to identify at least three hepatic cytosolic isoenzymes in the rat (Hiratsuka *et al.*, 1990). The major form which is active with this substrate has been purified and designated GST YrsYrs. It was found to posses enzymic activity towards a number of reactive sulphate esters including 1-menaphthyl sulphate, a compound which had been used earlier for the identification of rat liver GST M (Gillham, 1973).

**Figure 1.06 Metabolic activation and inactivation of 5-methylchrysene**

5-Hydroxymethylchrysene (5-HCR) is a major metabolite of 5-methylchrysene in rat liver. In the presence of a 3′-phosphoadenosine 5′-phosphosulphate (PAPS)-generating system this compound may be activated to the highly mutagenic sulphate conjugate, 5-HCR sulphate, by rat liver sulphotransferase. The conjugation of GSH to 5-HCR sulphate mediated by GST present in rat liver cytosol prevents the formation of mutagenic purine base adducts.

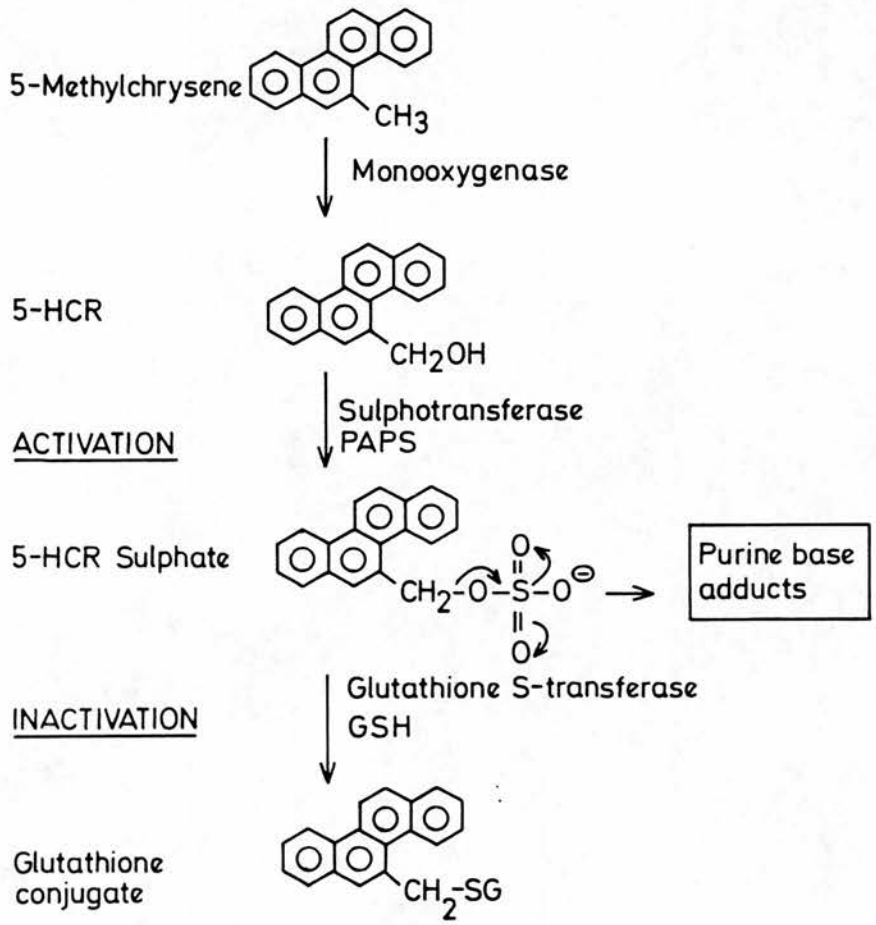

5-Methylchrysene
CH3
Monooxygenase
5-HCR
CH2OH
Sulphotransferase
PAPS
ACTIVATION
5-HCR Sulphate
CH2-O-S-O
O
O
Purine base adducts
Glutathione S-transferase
GSH
INACTIVATION
Glutathione conjugate
CH2-SG

**Figure 1.06**

### 1.05b The conjugation of glutathione with therapeutic drugs

A number of alkylating agents, used as chemotherapeutic drugs, have been shown to serve as GST substrates. These include the nitrogen mustards melphalan (Dulik and Fenselau, 1987), chlorambucil and cyclophosphamide (Colvin and Hilton, 1988) and the nitrosourea 1,3-bis(2-chloroethyl)-1-nitrosourea (Smith *et al.*, 1989). The emergence of drug resistant cell populations following treatment with these and other agents is often a major obstacle to effective cancer chemotherapy. The cytotoxic effect of many of the anti-cancer drugs is mediated by the action of drug metabolising enzymes, which include the GST. Changes in the levels of expression of these enzymes are likely, therefore, to be an important mechanism which leads to alterations in the sensitivity of cells to cytotoxic chemicals.

Many mammalian cell models have been studied where an increased resistance to cytotoxic insult is accompanied by the over-expression of GST enzymes (for a review see Hayes and Wolf, 1988). In one such study rat Mu-class GST were shown to be elevated in a rat brain tumour cell line which was resistant to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). When purified rat Alpha-, Mu- and Pi-class GST were analysed for their ability to detoxify BCNU, Mu-class isoenzymes were found to be the best catalysts of the denitrosation reaction (Smith *et al.*, 1989). Recently, in an attempt to obtain more direct evidence on the involvement of GST in drug resistance, some workers have performed cDNA transfection experiments. One of these studies showed that the transfection of cDNA's encoding human Alpha- and Pi-class GST confers resistance to both chlorambucil and the anthracycline adriamycin to yeast cells (Black *et al.*, 1990). A similar approach has also demonstrated that mammalian cells transfected with full length cDNA's for human Pi-class and rat Alpha- and Mu-class GST provide a decrease in sensitivity to the nitrogen mustards chlorambucil and melphalan (Puchalski and Fahl, 1990).

## 1.06 Endogenous substrates

Oxidative metabolism in aerobic organisms results in the production of a number of highly reactive molecular species. It has, therefore, been proposed that glutathione and glutathione-dependant enzymes originally evolved in order to deal with the inactivation of the toxic products of oxygen metabolism (Mannervik, 1986). Some of the endogenous electrophiles which have been investigated as potential natural substrates for the GST are discussed below.

## 1.06a GST and protection against lipid and DNA peroxidation

The peroxidation of lipid biomembranes occurs following the attack of free radicals upon the polyunsaturated fatty acyl moieties. In the presence of oxygen this can lead to chain propagation reactions involving lipid peroxy radicals and hydroperoxides. If this process is unimpeded the consequences include the release of toxic breakdown products and the eventual destruction of the lipid component of biological membranes (Slater, 1984). This process can be prevented by the intervention, at various points, of a number of different enzymes, including selenium-dependant glutathione peroxidase, catalase and superoxide dismutase.

A possible protective role of GST isoenzymes, that involves their selenium-independent glutathione peroxidase activity, has been investigated. Some workers have demonstrated that the consecutive action of the enzyme phospholipase $A_2$, which releases fatty acyl hydroperoxides from peroxidized phospholipids, and certain rat Alpha-class GST, which reduce these free fatty acid hydroperoxides, is sufficient for the inhibition, *in vitro*, of microsomal lipid peroxidation (Tan *et al.*, 1984). It was subsequently reported that a number of purified rat GST had activity towards hydroperoxides of linoleic acid and arachidonic acid, two polyunsaturated fatty acids which are abundant in biological membranes (Ketterer *et al.*, 1987). Rat Alpha- and Pi-class GST and an isoenzyme called GST 5-5, which has recently been ascribed to the Theta-class, were shown to have the highest specific activities towards these fatty acid hydroperoxides.

One class of highly toxic compounds which are by-products of lipid peroxidation are the 4-hydroxy unsaturated aldehydes or 4-hydroxyalkenals (Esterbauer *et al.*, 1982). A number of mammalian GST isoenzymes are highly efficient in the detoxification of these compounds (Danielson *et al.*, 1987). The most active of the GST towards the biologically active compound 4-hydroxynonenal is a rat Alpha-class GST called YkYk (Jensson *et al.*, 1986). However, certain rat and human Mu-class GST have also been shown to express a significant amount of activity towards this substrate (Alin *et al.*, 1985b; Danielson *et al.*, 1987). Cholesterol $\alpha$ epoxide is a further example of a by-product of lipid peroxidation which is a possible natural substrate for the GST. The conjugation of glutathione to this weakly mutagenic compound, is catalysed by rat Alpha-class GST (Meyer and Ketterer, 1982).

**Figure 1.07 Endogenous GST substrates**

Panels a, b and c depict the following GST catalysed reactions:

a) Reduction of 9-hydroperoxy-linoleic acid

b) Glutathione conjugation of 4-hydroxyalkenal

c) Reduction of 5-hydroperoxymethyl uracil

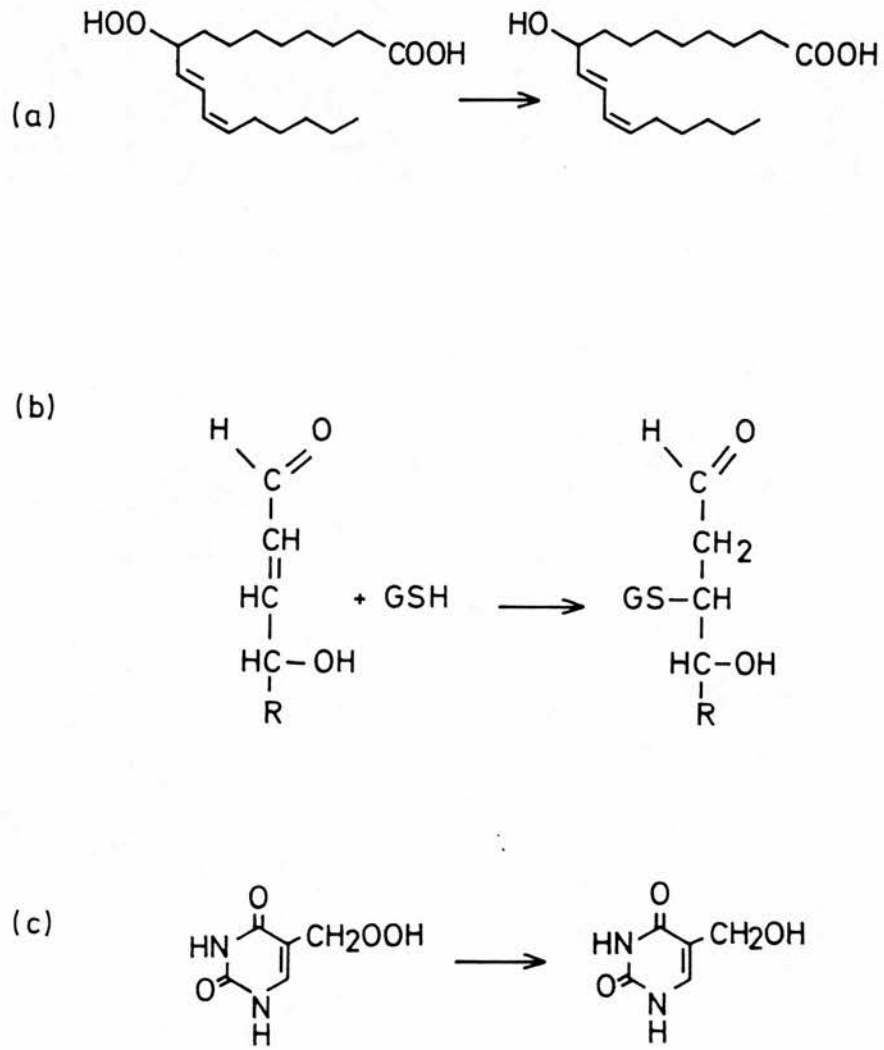

(a)
HOO
COOH
HO
COOH
(b)
H
O
C
CH
HC
HC – OH
R
+ GSH
H
O
C
CH2
GS – CH
HC – OH
R
(c)
O
HN
O
N
H
CH2OOH
O
HN
O
N
H
CH2OH

**Figure 1.07**

DNA is also susceptible to free radical attack during oxidative stress. The participation of GST isoenzymes in the detoxification and repair of the potentially mutagenic radical damage to DNA has been studied by a number of workers. Both thymine hydroperoxides and DNA peroxidized by exposure to ionising radiation in the presence of oxygen have been shown to be GST substrates (Tan *et al.*, 1986, 1988). However, these workers reported that the specificity of rat GST isoenzymes for the peroxidized DNA was different from that towards the free thymine hydroperoxide 5-hydroxymethyl uracil. Certain rat Alpha- Mu- and Pi-class enzymes had a high activity towards 5-hydroxymethyl uracil whereas, two different Mu-class GST and the Theta-class GST 5-5 demonstrated activity towards DNA hydroperoxide (Tan *et al.*, 1988). Some of the GST catalysed reactions described above are depicted in Figure 1.07.

1.06b Other endogenous reactions catalysed by GST

GST isoenzymes have been shown to catalyse *in vitro* a number of reactions which are of physiological importance. For example the product of the GST-mediated isomerization reaction involving $\Delta^5$ androstene-3,17-dione (see Figure 1.03d) is a precursor for testosterone.

The biologically active cysteine-containing leukotriene $C_4$, $D_4$ and $E_4$ are presumed to mediate anaphalactic and allergic reactions. Leukotriene $C_4$ is formed from leukotriene $A_4$ following the conjugation of glutathione to an epoxy group on the parent compound. The identity of the GST responsible for this leukotriene C synthase activity has not been fully established. A rat microsomal GST present in basophilic leukaemia cells and purified human cytosolic GST from classes Alpha, Mu and Pi have all been shown to posses catalytic activity towards leukotriene $A_4$ (Bach *et al.*, 1984; Soderstrom *et al.*, 1985). More recently, however, evidence for the existence of a distinct microsomal enzyme, with a high leukotriene C synthase activity, in mastocytoma cells from the mouse has also been presented (Soderstrom *et al.*, 1988).

The hydroxy endoperoxide prostaglandin (PG) $H_2$ may undergo three different glutathione-dependant conversions in order to form biologically active compounds. The precursor molecule can be reduced to $PGF_{2\alpha}$ or isomerized to $PGD_2$ or $PGE_2$. All three reactions are catalysed by rat cytosolic GST *in vitro* (Christ-Hazelhof *et al.*, 1976; Chang *et al.*, 1987; Meyer and Ketterer, 1987).

# Chapter 2 Glutathione S-transferase isoenzymes

## 2.01 Multiple GST forms

### 2.01a Cytosolic classes of GST

In all of the mammalian species examined to date the GST are represented by a large number of isoenzymes. Historically, the multiple forms of GST which are present in rat and human cytosol were each assigned to several different groups or classes. The rat enzymes have been classified, according to their immunochemical properties, as belonging to groups I, II and III (Hayes and Mantle, 1986a), whereas, the human GST were originally subdivided, on the basis of their charge, into the basic, near-neutral and acidic forms (Mannervik, 1985).

Mannervik and co-workers undertook a comparison of the catalytic properties, immunochemical cross-reactivities and N-terminal amino acid sequences of the major cytosolic enzymes from several mammalian species (Mannervik *et al.*, 1985). This work clearly demonstrated the existence of relationships between the groups of isoenzymes previously identified in rodents and humans, and led to the general acceptance of a species-independent classification system. Three evolutionary classes of GST, designated Alpha, Mu and Pi, were identified, and these represent the rat group I, II and III enzymes and the human basic, near-neutral and acidic GST, respectively. Recently, several rat isoenzymes have been described which belong to a fourth cytosolic class of GST, which has been called Theta by some workers (Meyer *et al.*, 1991). Detailed information on the existence of Theta-class GST in species other than the rat has yet to be obtained.

### 2.01b Rat GST nomenclature

The cytosolic GST exist as homodimeric and heterodimeric proteins which have physical and enzymatic properties which reflect their subunit composition. Therefore, the systems of nomenclature which have been favoured by most workers in this field are those which take account of the quaternary structure of the enzymes. Two classification systems are currently used to describe the rat cytosolic GST, one based on the relative mobilities of

the individual subunits on SDS/PAGE (Bass *et al.*, 1977), and a second in which the subunits have been assigned Arabic numerals in chronological order of their isolation and characterization (Jakoby *et al.*, 1984).

Bass *et al.* (1977) originally identified three types of GST subunit present in rat liver cytosol, which were termed Ya, Yb and Yc in the order of their relative mobilities on SDS/PAGE. Subsequent studies into the GST content of rat liver and other tissues have revealed the existence of other subunit types, including Yf, Yk, Yn, Yl, Yo and Yrs (Hayes, 1986, 1988; Hayes and Mantle, 1986a, 1986b; Ishikawa *et al.*, 1988; Hiratsuka *et al.*, 1990). Improvements in protein purification and the application of molecular cloning techniques has also revealed the existence of multiplicity within certain GST subunit types. This multiplicity has been documented for the Ya-type subunits (Rothkopf *et al.*,1986; Hayes *et al.*, 1990), Yc-type subunits (Hayes *et al.*, 1991), Yb-type subunits (Hayes, 1984; Ding *et al.*, 1986) and Yn-type subunits (Ishikawa *et al.*, 1988). In addition, several rat GST have been described which have not, as yet, received a "Y" designation. These include the Theta-class GST 5-5 and GST 12-12, which were previously co-purified and designated GST E (Meyer *et al.*, 1984, 1991).

The inter-relationship between the "Y" designations and the numerical designations of the multiple GST subunits, together with their species-independent class designations, are shown in Table 2.01. The system of nomenclature used when discussing rat GST in this thesis will be that which describes the subunit types according to their mobility on SDS/PAGE (Hayes, 1986; Hayes and Mantle, 1986a, 1986b).

### 2.01c Human GST nomenclature

Several systems of nomenclature have been adopted by workers in different laboratories for the classification of the human GST isoenzymes. Kamisaka *et al.* (1975) originally described five basic (Alpha-class) forms of GST in human liver which were assigned Greek alphabetical symbols ($\alpha$, $\beta$, $\gamma$, $\delta$ and $\epsilon$). Following the identification of near-neutral (Mu-class) GST in liver, and acidic (Pi-class) GST in placenta, the additional forms were also designated by the Greek symbols $\mu$, $\psi$, $\phi$ and $\pi$ (Guthenberg *et al.*, 1979; Warholm *et al.*, 1981; Singh *et al.*, 1987a; Stockman and Hayes, 1987; Hayes, 1989).

Further studies on the hepatic basic GST determined the subunit composition of these enzymes (Stockman *et al.*, 1985). This allowed a system of nomenclature, which is based

**Table 2.01 Nomenclatures for the rat cytosolic GST subunits**

| Class | Subunit[1-6] | Subunit $M_r$[1-7] | Numerical Designation[7-14] |
|---|---|---|---|
| Alpha | $Ya_1$ | 25500 | 1 |
| | $Ya_2$ | 25500 | 1 |
| | $Yc_1$ | 27500 | 2 |
| | $Yc_2$ | 25800 | 10 |
| | Yl | 25700 | N.I. |
| | Yk | 25000 | 8 |
| Mu | $Yb_1$ | 26300 | 3 |
| | $Yb_2$ | 26300 | 4 |
| | $Yn_1$ or $Yb_3$ | 26000 | 6 |
| | $Yn_2$ | 26000 | 9 |
| | Yo | 26500 | 11 |
| Pi | Yf or Yp | 24800 | 7 |
| Theta | Yrs | 26000 | N.I. |
| | N.I. | 28500 | 5 |
| | N.I. | unknown | 12 |

References:


1. Hayes *et al.*, 1990
2. Hayes and Mantle, 1986a
3. Hayes *et al.*, 1991a
4. Hayes, 1988
5. Ishikawa *et al.*, 1988
6. Hiratsuka *et al.*, 1990
7. Meyer *et al.*, 1984
8. Jakoby *et al.*, 1984
9. Meyer *et al.*, 1985
10. Guthenberg *et al.*, 1985
11. Jensson *et al.*, 1986
12. Ketterer *et al.*, 1988
13. Kispert *et al.*, 1989
14. Meyer *et al.*, 1991


Abbreviations: N.I., not included

upon the quaternary structure of the proteins, to be proposed for the human Alpha-class GST. This system describes three enzymes formed by the combination of two structurally and immunologically distinct subunits called $B_1$ and $B_2$ (Stockman *et al.*, 1985, 1987; Hayes *et al.*, 1989). The homodimeric enzyme GST $B_1B_1$ represents GST $\epsilon$, and the heterodimer $B_1B_2$ is equivalent to GST $\delta$. However, the relationship of $B_2B_2$ to the enzymes described by Kamisaka *et al.* (1975) is less clear; this homodimeric enzyme is probably equivalent to GST $\alpha$, $\beta$ and $\gamma$, three forms which may have arisen following the autoxidation of a single gene product.

The human GST have also been named according to their pI value. Del Boccio *et al.* (1987) have described a very basic enzyme, called GST 9.9, purified from human skin which has an identical N-terminal amino acid sequence to that of the rat Alpha-class enzyme GST $Yc_1Yc_1$.

Other workers, who have taken a genetic approach to the classification of the human GST have numbered the enzymes, according to their gene loci, using evidence obtained from zymogram analysis (Board, 1981; Strange *et al.*, 1984). This approach has given rise to the designation GST1, GST2 and GST3 , which are the loci encoding enzymes of the Mu-class, Alpha-class and Pi-class GST respectively. Similar investigations into a number of different human tissues indicated the existence of at least three more gene loci termed GST4, GST5 and GST6 (Laisney *et al.*, 1984; Suzuki *et al.*, 1987). Later reports have shown that GST4 and GST6 encode enzymes that belong to the Mu evolutionary class (Board *et al.*, 1988; Suzuki *et al.*, 1991).

A list of the major human cytosolic GST described in the literature is given in Table 2.02.

## 2.02 Human Alpha-class GST

The expression of human GST isoenzymes is subject to both inter-organ and inter-individual variation (Laisney *et al.*, 1984; Strange *et al.*, 1984; Suzuki *et al.*, 1987; Corrigal and Kirsch, 1988). The highest levels of Alpha-class GST are found in liver, however, significant amounts of the basic enzymes are also present in human testis, kidney and adrenal glands (Tateoka *et al.*, 1987; Singh *et al.*, 1987b; Aceto *et al.*, 1989; Meikle *et al.*, 1992).

**Table 2.02 Nomenclatures for the human cytosolic GST isoenzymes**

| Class | Isoenzyme[1-7] | Locus[7-9] |
|---|---|---|
| Alpha | $B_1B_1$ | GST2, 1 |
| | $B_1B_2$ | GST2, 1-2 |
| | $B_2B_2$ | GST2, 2 |
| | GST 9.9 | unknown |
| Mu | $\mu$ | GST1, 2 |
| | $\psi$ | GST1, 1 |
| | $\phi$ | unknown |
| | GST4 | GST4 |
| Pi | $\pi$ | GST3 |

References:


1. Stockman *et al.*, 1985
2. Del Boccio *et al.*, 1987
3. Warholm *et al.*, 1983
4. Hayes, 1989
5. Stockman and Hayes, 1987
6. Guthenberg *et al.*, 1979
7. Board *et al.*, 1988
8. Board, 1981
9. Strange *et al.*, 1984


A number of workers have purified the hepatic Alpha-class GST and shown that they are represented by multiple isoenzymes. Kamisaka *et al.* (1975) originally observed five basic forms of GST ($\alpha$-$\epsilon$) which had different pI values but were indistinguishable by other criteria. It was, therefore, proposed that these enzymes were charge isomers formed as a result of deamination *in vivo* of a single gene product. Subsequent studies have shown, however, that the basic forms of GST present in human liver comprise two immunochemically and catalytically distinct subunits (Stockman *et al.*, 1985, 1987; Soma *et al.*, 1986). These subunits may combine to form two homodimeric enzymes, which have been designated GST $B_1B_1$ and GST $B_2B_2$, and one heterodimeric enzyme, called $B_1B_2$ (Stockman *et al.*, 1985, 1987). In contrast to these reports, Vander Jagt *et al.* (1987) have

described the isolation of 13 Alpha-class enzymes from a number of different liver specimens. However, it is likely that these forms do not represent separate enzymes but are multiple forms generated by the purification strategy (see Hayes *et al.*, 1989).

Several groups of workers have investigated the GST isoenzyme content of different tissues using starch-gel electrophoresis combined with an activity stain. This type of zymogram analysis also revealed the existence of three electrophoretically distinct cationic GST enzymes in human liver (Board, 1981; Strange *et al.*, 1984). These three forms were originally thought to be the product of one gene locus which was designated GST2. The reports published at this time, however, presented conflicting evidence as to whether the separate enzymes which were observed had arisen following the post-translational modification of a single gene product (Laisney *et al.*, 1984; Strange *et al.*, 1984) or as to whether they were formed from the combination of two allelic products (Board, 1981).

Molecular cloning studies, which allowed the investigation of human Alpha-class GST at a nucleic acid level, were also undertaken during this period. Tu and his colleagues described two separate full-length cDNA clones, called $GTH_1$ and $GTH_2$, which both encoded human Alpha-class GST subunits (Tu and Quain, 1986; Rhoads *et al.*, 1987). Independently, Board and Webb (1987) cloned a cDNA which encoded the same GST subunit as the cDNA clone described by Tu and Quain (1986). An analysis of the deduced amino acid sequences from these two human Alpha-class genes indicates that they encode two highly homologous subunits which differ in only 11 amino acids. A direct molecular relationship between the human Alpha-class cDNA clones described in the literature and the basic isoenzymes which may be purified from human liver was established following protein sequencing experiments. Hayes *et al.* (1989) showed that the amino acid sequences obtained from CNBr-derived peptides from GST subunits $B_1$ and $B_2$ were virtually identical to the deduced amino acid sequences of the cDNA clones $GTH_1$ and $GTH_2$ (Rhoads *et al.*, 1987), respectively.

At the present time it is unambiguous that there are at least two Alpha-class GST subunits which are the products of two separate gene loci (Board and Peirce, 1987; Hayes *et al.*, 1989). The chromosomal location of the genes which encode these human Alpha-class enzymes has been investigated using *in situ* hybridization techniques. Separate studies have indicated that the genes are clustered on the short arm of chromosome 6 at band p12 (Board and Webb, 1987; Chow *et al.*, 1988).

The possibility that there are additional human Alpha-class GST has also been the subject of investigation. One group of workers have documented the existence of an Alpha-class isoenzyme which is distinct from the hepatic GST. Del Boccio *et al.* (1987) purified a very basic GST from human skin which has a close relationship to the rat GST $Yc_1Yc_1$ (or 2-2). It is also of interest that the polymorphic expression of a similar GST has been observed in human prostate (Di Illio *et al.*, 1990). At a nucleic acid level, Southern blotting experiments, using a cDNA encoding the GST $B_1$ subunit, have revealed hybridization patterns that indicate that there may be multiple Alpha-class genes in the human genome (Board and Webb, 1987).

## 2.03 Human Mu-class GST

In man, the GST which comprise the Mu evolutionary class have been the subject of particular interest due to the polymorphic expression of certain of the isoenzyme forms. However, the multiplicity of the Mu-class GST, together with the variability in their expression has given rise to some confusion in the literature. Workers in this field have used a number of different approaches in order to analyze these isoenzymes. Some have studied the levels of GST expression in various human tissue extracts obtained from large populations using zymogram analysis, whereas others have attempted to purify and characterize the individual isoenzymes. A more recent approach has involved the application of molecular cloning techniques to the study of the Mu-class genes.

### 2.03a Genetic population studies

In an early investigation into the genetic basis of the expression of multiple GST isoenzymes in man, Board (1981) used zymogram analysis to examine the enzyme levels in liver cytosol extracts from a large number of individuals. This electrophoretic analysis indicated that the hepatic GST were the products of three separate gene loci, termed GST1, GST2 and GST3. Subsequent studies have demonstrated that the GST1 enzymes fall within the Mu-class (Suzuki *et al.*, 1987; Board *et al.*, 1988).

The initial electrophoretic study undertaken by Board (1981) showed that the products of the GST1 locus were represented by three activity bands which were subject to phenotypic variation. The most acidic of these enzymes was termed GST1 type 1 and the least acidic was termed GST1 type 2. These workers proposed that the isoenzyme with an intermediate mobility was a heterodimeric protein formed by the combination of GST1

type 1 and type 2 subunits. This type of analysis also revealed that a large number of individuals did not express any of the GST1 isoenzymes. A statistical analysis of the data obtained indicated that the different phenotypes observed were attributable to different combinations of three autosomal alleles, including a null, at a single gene locus.

Other workers subsequently confirmed this genetic model and extended this type of investigation to include the analysis of GST activity in tissues other than liver (Strange *et al.*, 1984; Laisney *et al.*, 1984). These studies showed that the GST1 phenotype was a constant individual characteristic which was observed in all of the tissues which had detectable levels of these enzymes. The report by Laisney *et al.* (1984) also included a description of two additional GST forms, called GST4 and GST5, which were present in certain extra-hepatic tissues. Both of these forms were thought to be the products of an independent gene locus. GST4 was shown to be abundant in skeletal muscle, but was also observed in a number of other tissues. GST5 was present in brain and also weakly observed in lung. No phenotypic variation in the expression of these two GST enzymes was reported by these investigators. This study also provided evidence for the existence of a molecular relationship between the GST1 and GST4 isoenzymes. These workers described electrophoretic bands which apparently represented heterodimeric proteins formed by the combination of GST1 subunits and GST4 subunits.

In a later study, which also incorporated the use of starch-gel electrophoresis, Suzuki *et al.* (1987) described enzymes which were similar to the GST4 and GST5 forms observed earlier, together with an additional form, termed GST6. A number of differences were apparent, however, between the results of this study and those of the previous report. Unlike Laisney *et al.* (1984), these workers did not detect heterodimeric GST formed as a result of the combination of GST1 and GST4 subunits. This investigation also provided evidence for an association between the expression of GST5 and GST1 type 1, which had not been observed by Laisney *et al.* (1984).

### 2.03b The polymorphic expression of Mu-class GST: An association with susceptibility to cancer?

A characteristic feature of human hepatic Mu-class GST is their high glutathione conjugating activity towards certain mutagenic epoxides, including benzo(a)pyrene-4,5-oxide and styrene 7,8-oxide. This has led to the suggestion that an enzyme responsible for this activity may play an important role in the protection against carcinogenic

compounds (Warholm *et al.*, 1983). The genetic polymorphism associated with human Mu-class isoenzymes has been well documented (Warholm *et al.*, 1980; Board, 1981; Strange *et al.*, 1984; Laisney *et al.*, 1984; Seidegard and Pero, 1985; Hussey *et al.*, 1987). The influence that this deficiency may have on an individuals susceptibility to the toxic effect of various xenobiotics has also been the subject of intense investigation.

A number of different workers have quantitated the expression of Mu-class GST using several different methods of analysis, including the starch-gel electrophoresis (Board, 1981; Strange *et al.*, 1984; Laisney *et al.*, 1984) and specific radioimmunoassay (Hussey *et al.*, 1987). A wide variation in the frequency of the nulled phenotype has been reported both within and between different racial groups (Table 2.03). One method which has been introduced for the detection of certain Mu-class enzymes is the measurement of GST activity towards *trans*-stilbene oxide in mononuclear leukocytes (Seidegard and Pero, 1985; Seidegard *et al.*, 1987). Seidegard and co-workers used this activity assay to asses the degree of protection afforded by the expression of Mu-class GST against the chemical carcinogens present in cigarette smoke. In two separate studies, these workers demonstrated significant differences between the distribution of *trans*-stilbene oxide activity in populations of control smokers and populations of lung cancer patients (Seidegard *et al.*, 1986, 1990). These investigators concluded that the expression of Mu-class GST may be used as a marker for susceptibility of an individual to lung cancer.

Recently, Strange *et al.* (1991) used a starch-gel zymogram approach to compare the frequency of the Mu-class GST null allele in controls and a group of patients with adenocarcinoma of the stomach or colon. In support of the findings of Seidegard *et al.* (1986, 1990), these workers also observed an increase in the frequency of the null phenotype in patients with carcinoma. However, there are several reports in the literature which contain contradictory evidence about the expression of Mu-class GST in patients with cancer. Peters *et al.* (1990) used an immunochemical detection method for the measurement of GST in mononuclear blood cells, and demonstrated that their were no significant differences in the distribution of Mu-class enzymes in patients with breast or colon cancer, as compared to controls.

The polymorphism associated with human Mu-class GST has also been investigated at a genomic level using Southern blotting analysis. Seidegard *et al.* (1988) showed that the null phenotype correlated with the absence of a hybridization to a cDNA probe encoding

**Table 2.03 The frequency of the Mu-class GST null phenotype**

| Population | No. | Mu-class GST null phenotype (%) | Study |
|---|---|---|---|
| Caucasian | 23 | 40 | Warholm *et al.*, 1980 |
| Caucasian | 40 | 65 | Board, 1981 |
| Caucasian | 49 | 41 | Strange *et al.*, 1984 |
| Caucasian | 56 | 43 | Laisney *et al.*, 1984 |
| Caucasian | 248 | 54 | Seidegard and Pero, 1985 |
| Caucasian | 42 | 45 | Hussey *et al.*, 1987 |
| Chinese | 96 | 58 | Board, 1981 |
| Indian | 43 | 35 | Board, 1981 |
| Japanese | 168 | 49 | Harada *et al.*, 1987 |

a hepatic Mu-class GST. In a recent study, one group of workers have used a genotyping assay, which involved this kind of analysis, to investigate the association between Mu-class GST polymorphism and the susceptibility of an individual to lung cancer (Zhong *et al.*,1991). In contrast to the findings of Seidegard *et al.* (1986, 1990), these workers saw similar distributions of the null allele in both a control population and a large group of lung cancer patients.

The data obtained from the investigations mentioned above are summarised in Table 2.04. The differences observed between the findings of these studies means that the consequences of the Mu-class GST polymorphism remain ill-defined.

2.03c Mu-class isoenzymes

A neutral GST that was distinct from the five basic forms (GST $\alpha$-$\epsilon$) purified earlier (Kamisaka *et al.*, 1975) was first identified in human liver in 1980 (Warholm *et al.*, 1980). This isoenzyme was characterized by its high activity towards the substrate *trans*-4-phenyl-3-buten-2-one and the fact that it was only present in the livers of some individuals. Warholm *et al.* (1981, 1983) subsequently purified and characterized this new form, called

**Table 2.04 The frequency of the Mu-class GST null phenotype in carcinoma patients**

| Population | No. | Mu-class GST null phenotype (%) | Study |
|---|---|---|---|
| Control smokers | 192 | 42 | Seidegard *et al.*, 1986,1990 |
| Lung cancer patients | 191 | 62 | |
| Controls | 49 | 41 | Strange *et al.*, 1984,1991 |
| Adenocarcinoma patients | 45 | 67 | |
| Controls | 64 | 38 | Peters *et al.*, 1990 |
| Carcinoma patients | 106 | 31 | |
| Control smokers | 225 | 42 | Zhong *et al.*, 1991 |
| Lung cancer patients | 228 | 43 | |

GST μ, and showed that it had both chemical and physical properties that distinguish it from hepatic GST α-ε and a placental form called GST π. The neutral enzyme was also found to be highly efficient at catalysing the conjugation of glutathione with the epoxides benzo(a)pyrene-4,5-oxide and styrene 7,8-oxide.

A number of isoenzymes which are related to GST μ have since been identified. A hepatic enzyme, called GST ψ, has been purified in several laboratories (Singh *et al.*, 1987a; Hayes *et al.*, 1987b), and it has been shown that this homodimeric protein has an identical N-terminal amino acid sequence to that of GST μ (Alin *et al.*, 1985a; Hayes, 1989). The expression of this additional Mu-class form in human liver is also subject to variation (Hussey *et al.*, 1986). It is now generally accepted that GST μ and GST ψ represent allelic variants encoded by the GST1 locus which was earlier identified by Board (1981) and Strange *et al.* (1984). Less information is available on the heterodimeric enzyme formed by the combination of "μ-type" and "ψ-type" subunits. The existence of this isoenzyme has been demonstrated by means of starch-gel electrophoresis and chromatofocusing (Strange *et al.*, 1984; Faulder *et al.*, 1987), however, so far, it has not been fully characterized. A third hepatic Mu-class enzyme, named GST φ, was identified by Stockman and Hayes (1987). This isoenzyme, which was present in only one of 20 livers examined by these workers, was shown to be immunologically related to GST μ and

GST $\psi$. It was, however, distinguished from these forms by its lower isoelectric point and the fact that, unlike GST $\mu$ and GST $\psi$, it has a blocked N-terminus (Stockman and Hayes unpublished information).

Several extra-hepatic GST, which were initially identified using starch-gel electrophoresis (see section 2.03a), were purified and characterized to a limited extent by Board and co-workers (Suzuki *et al.*, 1987). An enzyme, called GST4, was isolated from human skeletal muscle and shown to be immunologically related to GST $\mu$, and to have N-terminal amino acid sequence homology with GST $\mu$ (Board *et al.*, 1988). Two further forms, termed GST5 and GST6, were purified from brain by this group of workers (Suzuki *et al.*, 1987). GST5 was shown to be a homodimeric enzyme that is structurally similar to the products of the GST1 locus. GST6, on the other hand, was an immunochemically distinct protein which contained two dissimilar subunits.

Others (Singh *et al.*, 1988) have isolated novel GST from skeletal muscle with N-terminal sequences which are similar to those of GST $\mu$. However, a major muscle form identified by these investigators, and called GST $\zeta$ ,was unrelated to Alpha-, Mu- or Pi-class GST and possessed a blocked N-terminus.

2.03d Mu-class GST genes

Human Mu-class genes have been located on both chromosome 1 and chromosome 3. DeJong *et al.* (1988) carried out *in situ* hybridization using a cDNA encoding a human hepatic Mu-class GST and showed that the human Mu-class genes were clustered in region p31 of chromosome 1. In contrast, using a panel of human-rodent somatic cell hybrids and two different rat Mu-class cDNA probes, Islam *et al.* (1989) assigned Mu-class genes to chromosome 3. The second group of workers postulated that they had mapped the Mu-class loci, GST4 and GST5, which had been previously identified by Laisney *et al.* (1984) and Suzuki *et al.* (1987).

Two cDNA clones encoding hepatic Mu-class isoenzymes have been isolated and sequenced by Seidegard *et al.* (1988) and DeJong *et al.* (1988). The deduced amino acid sequences from these two clones differ at one residue as a result of a single base substitution (C→G) in the coding region of the cDNA. At amino acid residue 172 the protein encoded by the clone isolated by DeJong *et al.* (1988) contains lysine, whereas, the cDNA described by Seidegard *et al.* (1988) specifies asparagine at this position. The

difference between these two protein sequences produces a variation in charge that is compatible with the difference observed between GST $\mu$ and GST $\psi$. Conformation that the proteins encoded by these two clones do in fact represent the allelic variants encoded by the GST1 locus was obtained recently following the heterologous expression of both of these cDNA sequences in *E.Coli* (Widersten *et al.*, 1991).

Following the hybridization of specific cDNA probes to human genomic DNA, two groups of workers have shown that the Mu-class GST null phenotype is associated with the absence of certain restriction fragments (Seidegard *et al.*, 1988; DeJong and Tu, 1990). This has led to the conclusion that the null allele at the polymorphic Mu-class GST locus is due to a gene deletion. Southern blotting analysis has also revealed a high level of complexity in the genomic organization of the human Mu-class genes. This type of analysis has indicated that there are at least six Mu-class genes present in the human genome (DeJong and Tu, 1990).

### 2.04 Human Pi-class GST

In man the Pi-class isoenzyme is widely distributed and represents the most thoroughly characterized extra-hepatic GST. Pi-class GST have been purified from a number of sources including placenta (Guthenberg *et al.*, 1979), lung (Koskelo *et al.*, 1981; Partridge *et al.*, 1984) and erythrocytes (Marcus *et al.*, 1978). Although the human Pi-class enzyme is now commonly referred to as GST $\pi$, the forms isolated from placenta, lung and erythrocytes were originally named GST $\pi$, GST $\lambda$ and GST $\rho$, respectively.

Some controversy has existed in the literature regarding the possible existence of more than one Pi-class GST. The acidic isoenzymes isolated from erythrocytes, lung and placenta have been shown to share immunological identity and have the same subunit $M_r$ value and isoelectric point (Koskelo, 1983; Howie *et al.*, 1988). However, using non-denaturing starch-gel electrophoresis Laisney *et al.* (1984) and Suzuki *et al.* (1987) have shown that an acidic isoenzyme present in erythrocytes has a different mobility to that of the GST3 enzyme observed in other tissues. Other workers have described two Pi-class isoenzymes present in skeletal muscle tissue which have minor differences in their isoelectric point (Singh *et al.*, 1988). It worth noting, however, that GST $\pi$, the skeletal muscle forms and the erythrocyte GST all share an identical N-terminal amino acid sequence (Alin *et al.*, 1985a; Suzuki *et al.*, 1987; Singh *et al.*, 1988).

At a nucleic acid level, a number of investigators have isolated cDNA clones encoding a human Pi-class GST (Kano *et al.*, 1987; Moscow *et al.*, 1988; Board *et al.*, 1989). A comparison between the deduced amino acid sequence of a human GST $\pi$ cDNA and that of the rat GST YfYf shows a very high level of homology (86%) and indicates that they represent corresponding enzymes in these species (Kano *et al.*, 1987).

The genomic GST $\pi$ gene has also been cloned and characterized by several workers (Cowell *et al.*, 1988; Morrow *et al.*, 1989). The chromosomal location of this gene was originally mapped to chromosome 11 by zymogram analysis of the GST isoenzymes expressed in somatic cell hybrids (Laisney *et al.*, 1984; Suzuki and Board, 1984). Subsequently, *in situ* hybridization experiments confirmed these earlier results and assigned the GST3 locus to 11q13 (Moscow *et al.*, 1988; Board *et al.*, 1989). The report published by Board *et al.* (1989), however, also contained evidence for the existence of a second human Pi-class gene in region q13-14 of chromosome 12. In this context, it is interesting that the amino acid sequence of a fatty acid ethyl ester (FAEE)-synthetase from human myocardium has been reported to be highly similar to, but not identical with, that of GST $\pi$ (Bora *et al.*, 1989a). This enzyme and a second related FAEE-synthetase from human heart have also been shown to have activity towards 1-chloro-2,4-dinitrobenzene (Bora *et al.*, 1989b). These results, therefore, suggest that additional Pi-class GST exist which may have an involvement in ethanol metabolism.

**2.05 Theta-class GST**

The existence of a fourth class of cytosolic GST has recently been recognised following the purification of several novel rat GST. Hiratsuka *et al.* (1990) identified at least three hepatic isoenzymes which were shown to posses catalytic activity towards reactive sulphate esters. These investigators purified one of these forms, designated GST YrsYrs, and demonstrated that it has structural, immunological and catalytic properties that distinguish it from Alpha- Mu- and Pi-class GST. In a subsequent study, Meyer *et al.* (1991) purified two enzymes from rat liver which are structurally related to GST YrsYrs. These two forms, designated GST 5-5 and GST 12-12, were previously isolated within a preparation called GST E (Fjellstedt *et al.*, 1973; Meyer *et al.*, 1984).

A human hepatic Theta-class isoenzyme, called GST $\theta$, was also purified and partially characterized by Meyer *et al.* (1991). This isoenzyme was particularly labile and represented only 0.003% of the soluble liver protein. The N-terminal amino acid sequence

of GST $\theta$ was shown to be highly homologous to those of the rat Theta-class isoenzymes GST 5-5 and GST 12-12. It is of interest, however, that electrophoretic analysis of the human enzyme indicated that this preparation contained two dissimilar subunits. Therefore, the quaternary structure of the protein remains to be elucidated.

## 2.06 Aims of the thesis

The major aims of the work described in this thesis were as follows:

i) To investigate the expression of extra-hepatic Mu-class GST in man.

ii) To purify and characterise the human Mu-class GST from skeletal muscle, testis and liver in order to establish the molecular relationships between the multiple Mu-class forms.

iii) To investigate the existence of Theta-class GST, with activity towards the substrate 1-menaphthyl sulphate, in human and mouse liver.

iv) To purify and characterise the human and murine Theta-class GST.

# PART II

# EXPERIMENTAL

# Chapter 3 Routine and analytical methods

## 3.01 Materials

### 3.01a Chemicals

*Aldrich Chemical Co., Gillingham, Dorset, UK*
*trans*-4-Phenyl-3-buten-2-one.

*BDH Chemicals Ltd., Poole, Dorset, UK*
Acetic acid; acrylamide; 2-amino-2-(hydroxymethyl)propane-1,3-diol(Tris); ammonium persulphate; ammonium sulphate; bromophenol blue; butan-1-ol; calcium chloride; 1-chloro-2,4-dinitrobenzene; Coomassie brilliant blue R250; disodium hydrogen orthophosphate; ethanol; glycerol; glycine; hydrochloric acid; hydrogen peroxide; 2-mercaptoethanol; methanol; N,N/-methylenebisacrylamide; orthophosphoric acid; potassium chloride; sodium azide; sodium chloride; sodium dodecyl sulphate; sodium hydroxide; 5-sulphosalicylic acid; N,N,N,/N/-tetramethyl ethylenediamide (TEMED); trichloroacetic acid.

*Bio-Rad Laboratories, Hemel Hempstead, Herts., UK*
Immuno-blot assay kit.

*Koch-Light Laboratories Ltd., Colinbrook, Berks., UK*
Cumene hydroperoxide; 1,2-dichloro-4-nitrobenzene.

*Pharmacia Limited, Milton Keynes, Bucks., UK*
Ampholine solutions; protein pI standards for isoelectric focusing.

*Rathburn Chemicals Ltd., Peeblesshire, Scotland, UK*
Acetonitrile; trifluoroacetic acid.

*Sigma Chemical Co. (London) Ltd., Poole, Dorset, UK*

Bis-tris; bromosulphophthalein; Coomassie brilliant blue G-250; cyanogen bromide; 1,2-epoxy-3-(*p*-nitrophenoxy)propane; ethacrynic acid; ethanolamine; ethylenediaminetetraacetic acid (EDTA); Freunds adjuvant (complete and incomplete); gelatin; glutathione (reduced form); glutathione reductase; iminodiacetic acid; nicotinamide adenine dinucleotide phosphate (reduced form, type X); *p*-nitrobenzyl chloride; Tween-20.

3.01b Equipment

*Anderman and Company Ltd., Kingston-upon-Thames, Surrey, UK*

Nitrocellulose paper.

*Elkay Laboratory Products (UK) Ltd., Basingstoke, Hants., UK*

Flow-rated pump tubing.

*Millipore (UK) Ltd., Harrow, Middlesex, UK*

Ultrafiltration membranes.

*Scientific Instruments Centre Ltd., London, UK*

Visking dialysis tubing.

3.01c Materials for chromatography

*Amicon Ltd., Stonehouse, Glos., UK*

Matrex™ Gel Orange A.

*Bio-Rad Laboratories, Hemel Hempstead, Herts., UK*

Bio-Gel HT-grade Hydroxyapatite; Bio-Gel HPHT h.p.l.c. grade hydroxyapatite column.

*Chromatography Services Ltd.,Merseyside, Cheshire, UK*

DEAE-Cellulose.

*Millipore, Harrow, Middlesex, UK*

μ Bondapak $C_{18}$ reverse-phase h.p.l.c. column.

*Pharmacia (Laboratory Separation Division), Milton Keynes, Bucks, UK*

Mono P HR 5/20 f.p.l.c. chromatofocusing column; Mono Q 5/5 f.p.l.c. anion exchange column; Polybuffer exchanger (PBE) 94.

*Sigma Chemical Co., (London) Ltd., Poole, Dorset, UK*

Epoxy-activated Sepharose 6B; Polybuffer 74 and 96; Sephadex G-100.

3.01d Reagents obtained from non-commercial sources

*Materials for chromatography*

The S-hexylglutathione was provided by Dr JD Hayes; S-hexylglutathione-Sepharose 6B affinity matrix was made, according to the method of Mannervik and Guthenburg, (1981), by coupling the $\gamma$-glutamyl moiety of S-hexylglutathione to epoxy-activated Sepharose 6B.

*GST substrates*

The GST substrate 1-menaphthyl sulphate was a gift from Dr F Oesch (Section on Biochemical Pharmacology, University of Mainz, Germany); the substrates 4-hydroxy-nonenal and 4-hydroxydecinal were generously provided by Dr H Esterbauer (Insitute of Biochemistry, University of Graz, Austria).

*Standards for electrophoresis*

Purified human GST isoenzymes $B_1B_2$ and $\mu$ and rat isoenzymes YoYo, YnYn and $Yb_1Yb_1$, which were used as standards during electrophoresis and Western blotting, were from Dr JD Hayes; human GST $\pi$ standard was a gift from Dr AF Howie.

*Antisera*

Antisera raised in rabbits against the human GST enzymes $B_1B_1$, $\mu$ and $\pi$ were provided by Drs GJ Beckett, JD Hayes and AF Howie (all of Department of Clinical Biochemistry, University of Edinburgh); rabbit antisera against the rat enzymes YoYo, YnYn and $Yb_1Yb_1$ were from Dr JD Hayes; antiserum raised in sheep against rat GST E was generously provided by Dr WB Jakoby (The National Institutes of Health, Bethesda, Maryland, USA).

3.01e Animals

Inbred C57BL6 mice were obtained from the Animal House, Department of Bacteriology, University of Edinburgh, where the animals were bred "in house". New Zealand White rabbits were obtained from Charles River Breeding Laboratories, UK.

## 3.02 General methods

3.02a Protein determination

Protein concentrations were measured using the method of Bradford (1976) adapted for use on the Cobas Fara (Roche Diagnostics, Welwyn Garden City, UK) centrifugal analyzer as described in detail by Howie, (1990).

3.02b Sodium determination

Sodium concentrations in the fractions obtained following column chromatography were estimated using flame photometry with a IL 343 photometer (Instrumentation Laboratory (UK) Ltd., Warrington, Cheshire, UK).

3.02c Enzymic activity assays

Enzymic activity assays for all substrates were carried out at 37 °C. In each case a non-enzymic rate was determined and subtracted from the rates measured in samples.

The measurement of enzymic activity with four substrates, 1-chloro-2,4-dinitrobenzene (CDNB), 1-menaphthyl sulphate (1-MS), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) and cumene hydroperoxide were carried out using methods adapted for use on a Cobas Fara centrifugal analyzer.

GST activity with CDNB as substrate was determined using the method described by Hayes and Clarkson (1982). Samples were pre-incubated with GSH and the reaction was started by the addition of CDNB. The reaction rates, calculated as ΔA/min/ml, were determined by an integral kinetic data program, which performed a linear regression analysis on 8 absorbance readings taken over 45 sec at 340nm.

The method of Gillham (1971) was used for the measurement of GST activity with the substrate 1-MS. Samples were also pre-incubated with GSH and the reaction initiated by the addition of the electrophilic substrate. Reaction rates were calculated using the data obtained from twenty absorbance measurements, at 298nm, taken at 30 sec intervals over 10 min. A pre-programmed conversion factor allowed the enzyme activity to be expressed as nmol 1-MS conjugated to GSH/min/ml.

GST activity towards the substrate EPNP was measured using the method of Habig and Jakoby (1981). The reaction was started by the addition of EPNP following an incubation of the sample with GSH. Fifty absorbance readings at 360nm were taken at 5 sec intervals over 250 sec. Enzyme activity was expressed as $\mu$mol GSH conjugated/min/ml.

Peroxidase activity, towards the substrate cumene hydroperoxide, was measured using an adaptation of the method of Wendel (1981). The first step involved a 5 min pre-incubation with NAPDH, glutathione reductase and GSH which was carried out in 60 mM Tris/HCl buffer pH 7.6 containing 0.12 mM EDTA and 1.0mM $NaN_3$. The reaction was initiated by the addition of cumene hydroperoxide. Absorbance readings, at 340 nm, were taken at 10 sec intervals for 1 min 40 sec allowing reaction rates to be calculated and expressed as $\mu$mol substrate reduced/min/L.

GST activity with the substrates *trans*-4-phenyl-3-buten-2-one, 1,2-dichloro-4-nitrobenzene, *p*-nitrobenzyl chloride and ethacrynic acid were all performed manually as described by Habig and Jakoby (1981). The assay for GST activity with bromosulphophthalein was also carried out manually using the method detailed by Habig *et al.* (1974a).

The substrates 4-hydroxynonenal and 4-hydroxydecinal were supplied at a concentration of 10 mg/ml in chloroform, and were stored at -20 °C. Prior to use, the chloroform solvent was removed by evaporation under nitrogen and the compounds were dissolved in distilled water. The concentration of the resulting solution was calculated using the molar extinction coefficient following an estimation of the maximum absorbance reading in the UV spectrum 200-300 nm. GST activities with these two substrates were determined manually using the method of Alin *et al.* (1985b).

The assay conditions and extinction coefficients for each of the enzyme assays described above are detailed in Table 3.01.

**Table 3.01 Conditions for enzymic activity assays**

| Substrate | [Substrate] (mM) | [GSH] (mM) | pH | Wavelength (nm) | Extinction coefficient ($mM^{-1}$ $cm^{-1}$) |
|---|---|---|---|---|---|
| 1-Chloro-2,4-dinitrobenzene | 1.0 | 2.0 | 6.5 | 340 | 9.6 |
| 1-Menaphthyl sulphate | 0.5 | 5.0 | 7.5 | 298 | 2.5 |
| 1,2-Epoxy-3-(*p*-nitrophenoxy) propane | 0.5 | 5.0 | 6.5 | 360 | 0.5 |
| Cumene hydroperoxide * | 1.2 | 1.0 | 7.6 | 340 | -6.2 |
| *trans*-4-Phenyl-3-buten-2-one | 0.05 | 0.25 | 6.5 | 290 | -24.8 |
| 1,2-Dichloro-4-nitrobenzene | 1.0 | 0.5 | 7.5 | 345 | 8.5 |
| *para*-Nitrobenzyl chloride | 1.0 | 5.0 | 6.5 | 310 | 1.9 |
| Ethacrynic acid | 0.2 | 0.25 | 6.5 | 270 | 5.0 |
| Bromosulphophthalein | 0.03 | 5.0 | 7.5 | 330 | 4.5 |
| 4-Hydroxyalk-2-enal | 0.1 | 0.5 | 6.5 | 224 | 13.75 |

* Final concentrations: NADPH, 0.25 mM, glutathione reductase, 1.0 unit/ml.

## 3.03 Analytical techniques

### 3.03a SDS/polyacrylamide-gel electrophoresis

SDS/Polyacrylamide-gel electrophoresis (SDS/PAGE) was performed, at room temperature, using the discontinuous buffer system of Laemmli (1970), in slab gels (0.01 cm x 16.5 cm x 18.0 cm) on a PROTEAN II electrophoresis system (Bio-Rad Laboratories, Hemel Hempstead, Herts UK) in the presence of 0.1% (w/v) SDS. The electrophoretic gels were made up of a "stacking gel" which was 2.5 cm long and a "resolving gel" which was 15 cm long. The "stacking gel" contained 3.6% (w/v) polyacrylamide and 0.01% (w/v) N,N$^{/}$-methylenebisacrylamide in 0.125 M Tris/HCl buffer pH 6.8 and the "resolving gel" contained 12% (w/v) polyacrylamide and 0.32% (w/v) N,N$^{/}$-methylenebisacrylamide ($C_{Bis}$ 2.6%) in 0.375 M Tris/HCl buffer pH 8.8.

Protein samples for electrophoresis were heated for 10 min at 90 °C in a solution containing 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue and 10% (v/v) glycerol. Samples were applied to wells within the "stacking gel" and subjected to electrophoresis at 50 mA (100 V) per gel through the "stacking gel", the current was then re-set to 50 mA (150 V) per gel before the sample entered the "resolving gel". Electrophoresis was terminated when the bromophenol blue marker reached 0.5 cm from the bottom of the "resolving gel". Gels were stained, for 20 min, using Coomassie brilliant blue R, 0.2% (w/v), in a solution of water: methanol: acetic acid (50: 50: 7 by vol) and de-stained by several changes of a solution containing water: methanol: acetic acid (88:7:5 by vol).

The molecular mass values for the novel GST subunits studied in this thesis were estimated using either rat or human GST enzymes for which the subunit Mr values have been determined previously (Hayes and Mantle, 1986b; Hayes, 1989).

### 3.03b Isoelectric focusing

Analytical isoelectric focusing was performed using an LKB 2117 Multiphore with an LKB 2103 power supply as described by the manufacturer (Pharmacia LKB Biotechnology AB, Bromma, Sweden). Electrofocusing was carried out in 5% (w/v) polyacrylamide slab gels (24 cm x 11.5 cm x 0.2 cm) containing 10% (v/v) glycerol and 5% Carrier Ampholine mixture covering the pH range 3.5-9.5. The electrode solutions were 1M NaOH (cathode)

and 1M $H_3PO_4$ (anode). Protein samples (5-10 $\mu$g) were applied to strips (1.0 cm x 1.0-2.0 cm) of Whatman No 1 filter paper positioned approximately 3.0 cm from the cathode. The gels were calibrated with protein pI standards which comprised trypsinogen (pI 9.30); lentil lectin (pI 8.65, 8.45, 8.15); horse myoglobin (pI 7.35, 6.85) human carbonic anhydrase B (pI 6.55); bovine carbonic anhydrase B (pI 5.85); $\beta$-lactoglobin A (pI 5.20); soybean trypsin inhibitor (pI 4.55); amyloglucosidase (pI 3.50).

Isoelectric focusing was carried out for 3.5 h at a constant power of 25 W; the sample application strips were removed after 1 h. The proteins were fixed in an aqueous solution of 3.5% (w/v) sulfosalicylic acid and 11.5% (w/v) trichloroacetic acid for 30 min and the gels were then washed in a de-staining solution of water: ethanol: acetic acid (67: 25: 8 by vol) for 30-60 min. Staining was achieved by immersing the gel in 0.2% (w/v) Coomassie brilliant blue R, dissolved in the de-staining solution, for 10 min at 60 °C followed by frequent changes of the de-staining solution.

3.03c Immunoblotting

Western blotting was performed by the method of Towbin *et al.* (1979) as described in detail by Hayes and Mantle (1986a). Protein samples, resolved by SDS/PAGE, were transferred to nitrocellulose paper using a Bio-Rad Trans-blot cell and Bio-Rad model 250/25 power supply (Bio-Rad Laboratories, Hemel Hempstead, Herts, UK). The polyacrylamide gels were first equilibrated, for 20 min, in 25 mM Tris/192 mM glycine buffer pH 8.3 followed by electrophoretic transfer, in the same buffer, performed at 0.25 A for 4 h at 10 °C. The nitrocellulose paper was then immersed in a blocking solution, containing 3% (w/v) gelatin and 500 mM NaCl in 20 mM Tris/HCl buffer pH 7.5, for 16 h at 20 °C.

After the blocking procedure, performed to reduce non-specific antibody binding, the nitrocellulose paper was incubated, for 3 h at 20 °C, in the primary antibody solution which comprised either a specific rabbit or sheep antiserum diluted, between 1:200-1:3000, depending on the titre of the antibody used, in 1% (w/v) gelatin in 20 mM Tris/HCl buffer pH 7.5 containing 500 mM NaCl and 0.05% (v/v) Tween 20. Visualisation of the proteins which cross-reacted with the specific antisera used was achieved, following extensive washing in the above buffer, using either a goat anti-(rabbit IgG) or a rabbit anti-(sheep IgG) antibody-horseradish-peroxidase conjugate immunoblot assay kit, as described by the manufacturer (Bio-Rad Laboratories, Hemel Hempstead, Herts., UK).

The immunogen, for the particular primary antibody used, was run alongside the other protein samples on each blot to serve as an internal control and molecular weight standard.

### 3.04 Protein sequencing

#### 3.04a CNBr cleavage

Portions of GST $N_2N_2$ (1.0 mg) and GST $N_3N_3$ (0.7 mg), purified under reducing conditions, were dialysed extensively against 10 mM ammonium acetate buffer pH 7.0 before freeze-drying. The lyophilised protein was then dissolved in 2.0 ml of aq. 70% (v/v) formic acid before the addition of a crystal of CNBr (approx. 25 mg). Incubation with CNBr was allowed to proceed in the dark for 20 h at room temperature (20 °C) before the reaction mixture was diluted with 15 volumes of distilled/deionised water (to terminate the cleavage process) before being freeze-dried.

A portion of GST $T_2T_2$ (60 $\mu$g), which had been purified under reducing conditions, was subjected to reverse-phase h.p.l.c. as described in section 4.05b. The protein in the peak recovered from the $\mu$ Bondapak $C_{18}$ column was freeze-dried before being dissolved in 0.15 ml of aq. 70% (v/v) formic acid containing approx. 2.0 mg of CNBr. Digestion was left to proceed in the dark for 20 h before the reaction mixture was diluted with 10 volumes of water and freeze-dried.

#### 3.04b Separation of peptides by reverse-phase h.p.l.c.

A Waters h.p.l.c. system (Waters Associates (Instruments), Northwich, Cheshire, UK) with a $\mu$ Bondapak $C_{18}$ column (10 $\mu$m particle size; 0.39 cm x 30.0 cm; Millipore, Harrow, Middlesex, UK) was employed for the separation of CNBr-cleaved peptides obtained from the Mu-class GST $N_2N_2$ and $N_3N_3$. The h.p.l.c. system comprised two model 510 pumps, a model 680 automated gradient controller, a model 481 Lambda-Max absorbance detector and a model U6K universal detector. The freeze-dried peptides were dissolved in 1.5 ml of aq. 0.1% (v/v) trifluoroacetic acid and centrifuged at 10,000 g for 5 min in a microcentrifuge, to remove particulate material, before application to the reverse-phase column. The gradient controller was programmed to allow the samples to be loaded isocratically onto the column over a period of 5 min. During this time the flow rate was increased linearly from 0.1-1.0 ml/min. A linear gradient of 0-70% (v/v) acetonitrile in aq.

0.1% (v/v) trifluoroacetic acid over 60 min was used to develop the column and resolve the peptide fragments. The $A_{220}$ of the eluate was monitored continuously allowing the peptide-containing peaks to be collected manually.

The separation of CNBr-cleaved peptides from GST subunit $T_2$ was performed by Margaret Daniel and Stephen D Peacock at the WELMET Protein Sequencing Laboratory, Department of Biochemistry, University of Edinburgh. The freeze-dried cleaved protein was re-dissolved in 0.2 ml of aq. 0.1% (v/v) trifluoroacetic acid and the peptides were resolved using a Applied Biosystems 130A Microbore Separation System with an Aquapore RP300 reverse-phase column (7 $\mu$m particle size; 2.1 mm x 30 mm; Applied Biosystems, Warrington, Cheshire, UK). The column was developed with a linear 0-70% acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid formed over 45 min.

### 3.04c Amino acid sequencing

Amino acid sequencing of either purified GST subunits or CNBr-cleaved peptides was performed by Andrew D Cronshaw, Margaret Daniel, Linda A Kerr and Stephen D Peacock at the WELMET Protein Sequencing Laboratory. Samples were first checked for purity on an Aquapore RP300 column (7 $\mu$m particle size; 2.1 mm x 30 mm). The column was developed with a linear 8-80% (v/v) acetonitrile gradient in aq. 0.1% trifluoroacetic acid and monitored at 220 nm.

Automated amino acid sequencing of the GST subunits and CNBr-cleaved peptides was performed on an Applied Biosystems 477A instrument with a 120A on-line phenylthiohydantoin analyzer. A glass-fibre filter disc was first loaded with polybrene (2 mg) which was pre-cycled three times before the protein sample to be analysed was loaded (approx. 1.5 nmol). Edman degradation of the N-terminus resulted in the cleavage of anilinothiazolinone derivatives from the peptide which were converted automatically to the more stable phenylthiohydantoin (PTH) forms. Separation of the PTH derivatives was achieved on an Applied Biosystems PTH $C_{18}$ (5 $\mu$m particle size; 2.1 mm x 220 mm) column developed, at 55 °C, with a linear 0-100% (v/v) acetonitrile gradient. The eluate from the column was monitored at 269 nm. Cysteine residues in the peptides were detected following vapour-phase pyridylation. This procedure is described in detail elsewhere (Hayes *et al.*, 1989).

### 3.05 Antibody production

Antisera against purified human GST $N_2N_2$ and $N_3N_3$ were produced in one and two female New Zealand White rabbits respectively. An aqueous solution of the purified GST was emulsified in an equal volume of Freunds complete adjuvant using a polytron homogenizer (Kinematica, Switzerland). Rabbits were injected subcutaneously, at multiple sites on the back, with the emulsion so that each animal received approx. 100 $\mu$g of protein. A booster injection, also containing 100 $\mu$g of purified GST, was prepared in Freunds incomplete adjuvant and given after six weeks. Rabbits were sacrificed after a further 10 days and blood was recovered by cardiac puncture. Serum was separated from the clot by centrifugation, at 3000 g for 15 min, and stored, in the presence of 0.1% (w/v) sodium azide, at -20 °C.

# Chapter 4 Purification methods

## 4.01 Tissue source

### 4.01a Human tissue

Human skeletal muscle, from both right and left psoas muscle, testis, cerebrum and liver were obtained *post mortem* by Dr DJ Harrison, Department of Pathology, University of Edinburgh, and stored at -80 °C before use. A macroscopic examination was performed at autopsy to establish that there was no abnormality in the tissue samples collected. Full details on the tissue samples used for the purification of GST isoenzymes are presented in Table 4.01.

### 4.01b Murine tissue

Male C57BL6 mice were killed at between 10-13 weeks old. The livers were removed and stored at -80 °C until used.

## 4.02 Preparation of cytosolic fractions

Tissue samples were allowed to thaw at room temperature and 25% (w/v) homogenates were then prepared in ice-cold buffer. The buffer used for homogenization was that used in the first chromatographic step of the purification procedure (see sections 4.07-4.11). All subsequent steps were performed either on ice or at 4 °C. Homogenates were centrifuged at 10,000 g, for 30 min, and the resulting supernatants were centrifuged at 100,000 g for 60 min. The supernatants obtained following centrifugation at 100,000 g were filtered through plugs of glass wool, to remove lipid material, and from this point are referred to as cytosol.

## 4.03 Ammonium sulphate precipitation

The precipitation of proteins by ammonium sulphate was performed at 4 °C (Dixon and Webb, 1979). Samples were taken gradually to 30% saturation with ammonium sulphate

**Table 4.01 Data on human tissue samples**

| Individual | Tissue | Sample number | Hours *post mortem* | Age | Sex |
|---|---|---|---|---|---|
| 1 | Skeletal muscle | M1 | <16 | 84 | female |
| 2 | Skeletal muscle | M2 | 4 | 60 | male |
| 3 | Skeletal muscle<br>Testis | M3<br>T3 | <16 | 68 | male |
| 4 | Skeletal muscle<br>Cerebrum<br>Liver | M4<br>C4<br>L4 | 13 | 73 | male |
| 5 | Liver | L5 | <16 | 49 | female |

and then incubated for 1.5 h. After centrifugation, at 10,000 g for 30 min, the resulting supernatant was taken to 90% saturation with ammonium sulphate and incubated for a further 1.5 h. The protein which precipitated between 30-90% saturation was recovered by centrifugation (10,000 g for 30 min) and resuspended in the appropriate buffer.

### 4.04 Conventional "open-column" chromatographic techniques

The chromatographic techniques described bellow were all performed at 4 °C.

#### 4.04a Affinity chromatography

Affinity purification of GST isoenzyme pools from cytosol was achieved using the S-hexylglutathione-Sepharose 6B affinity matrix described by Mannervik and Guthenberg (1981). Cytosolic fractions, which had been dialysed extensively against 20 mM Tris/HCl buffer, pH 7.8 containing 200 mM NaCl and 0.5 mM dithiothreitol, were applied to columns (1.6 cm x 30 cm) of S-hexylglutatione-Sepharose 6B which had been equilibrated in the same buffer. After washing with about 800 ml of the equilibration buffer the bound material was eluted by 5 mM S-hexylglutathione in the same buffer. The protein containing fractions, established by absorbance at 280 nm, were pooled prior to either analysis or further purification steps.

#### 4.04b Dye-ligand chromatography

Chromatography on Matrex™ Gel Orange A was used during the purification of a human Theta-class GST. The sample was dialysed against two changes, each of two litres, of 20 mM sodium phosphate buffer, pH 6.8, containing 1 mM EDTA and 0.5 mM dithiothreitol before application to a pre-equilibrated column (1.6 cm x 26 cm) at a flow rate of 34 ml/h. Following a wash of approx. 200 ml of the equilibration buffer the column was developed with a non-linear gradient of 0-1.0 M KCl in the sodium phosphate buffer. GST activity towards the substrates CDNB and 1-MS as well as glutathione peroxidase activity towards the substrate cumene hydroperoxide and absorbance at 280 nm were measured in each of the fractions (3.4 ml) collected.

### 4.04c Hydroxyapatite chromatography

*Human GST*

Hydroxyapatite chromatography, used during the purification of human Mu-class GST, was performed on a column of Bio-Rad HT grade hydroxyapatite (1.6 cm x 20 cm) which had been equilibrated with 10 mM sodium phosphate buffer pH 7.0, containing 0.5 mM dithiothreitol. The sample, previously dialysed against the same buffer, was applied to the column at a flow rate of 14 ml/h. Following washing, with approx. three column volumes of the equilibration buffer, the column was developed with a linear gradient (350 ml) of 10-250 mM sodium phosphate buffer, containing 0.5 mM dithiothreitol throughout. The fractions collected (2.3 ml) were monitored for GST activity towards CDNB and absorbance at 280 nm.

Human Theta-class GST were purified using a larger column (3.3 cm x 45 cm) of Bio-Rad HT grade hydroxyapatite which had been equilibrated in 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.5 mM dithiothreitol. The sample was applied as described above and the column developed using a non-linear 10-250 mM sodium phosphate gradient. The fractions obtained (4.7 ml) were examined for activity towards CDNB, 1-MS and cumene hydroperoxide as well as absorbance at 280 nm.

*Murine GST*

Chromatography of murine Theta-class GST on hydroxyapatite was carried out using a column of Bio-Rad HT grade hydroxyapatite (1.6 cm x 30 cm) which had been equilibrated in 10 mM sodium phosphate buffer pH 7.0, containing 1 mM EDTA and 2 mM 2-mercaptoethanol. The sample, which had been previously dialysed against the same buffer, was applied at a flow rate of 14 ml/h and the column was developed using a linear gradient (350 ml) of 10-250 mM sodium phosphate, containing 1 mM EDTA and 2 mM 2-mercaptoethanol. The fractions collected (2.3 ml) were monitored for activity towards CDNB, 1-MS, cumene hydroperoxide and absorbance at 280 nm.

### 4.04d Chromatofocusing

Conventional chromatofocusing of human Mu-class GST isoenzymes was performed, within the pH range 8.5-5.5, using a column (1.6 cm x 32 cm) containing polybuffer exchanger PBE 94, pre-equilibrated in 25 mM Tris/$CH_3COOH$ buffer pH 8.9. The pH

gradient was formed using polybuffer 96 diluted 1:10 (v/v) in water and adjusted to pH 5.5 with acetic acid. Immediately before chromatofocusing of the sample, 5 ml of the pH 5.5 eluant buffer was applied to the column to initiate the formation of the pH gradient. The sample, dialysed extensively against the equilibration buffer, was then applied at a flow rate of 34 ml/h and the column developed using the polybuffer pH 5.5 eluant. Fractions (3.4 ml) were collected and protein concentrations and GST activity, with CDNB as substrate, were measured. The pH was determined, at 4 °C, in every fifth fraction.

Murine Theta-class GST were subjected to chromatofocusing in the pH range 9.5-6.5 using a column (1.6 cm x 30 cm) of polybuffer exchanger PBE 94, equilibrated in 25 mM ethanolamine/HCl buffer, pH 9.5, containing 2 mM 2-mercaptoethanol. The partially purified GST were dialysed against two changes, each of two litres, of 25 mM ethanolamine/HCl buffer, pH 8.5, containing 2 mM 2-mercaptoethanol, before application to the column at a flow rate of 34 ml/h. A pH gradient was developed using polybuffer 96 diluted 1 in 10 (v/v) with water and adjusted to pH 6.5 with HCl, containing 2 mM 2-mercaptoethanol. The GST activity towards CDNB and 1-MS, protein concentrations and the pH at 4 °C were measured in the fractions collected.

### 4.04e Ion-exchange chromatography

Anion-exchange chromatography, using DEAE-cellulose, of murine and human Theta-class GST, was performed on columns of DE-52 (4.4 cm x 90 cm) equilibrated in 10 mM Tris/HCl buffer pH 8.25, containing 1 mM EDTA and either 2 mM 2-mercaptoethanol or 0.5 mM dithiothreitol respectively. Following application of sample, at a flow rate of 46 ml/h, the column was washed with the equilibration buffer before being developed with a non-linear gradient (400 ml mixing reservoir) of 0-300 mM NaCl in the same buffer. Fractions (11.5 ml) were collected and the GST activity, with 1-MS and CDNB as substrates, and the absorbance at 280 nm were measured.

### 4.04f Gel filtration

Gel filtration of GST enzymes was carried out on a Sephadex G-100 column (4.4 cm x 90 cm) equilibrated with 10 mM sodium phosphate buffer pH 7.0, containing 1 mM EDTA and 2 mM 2-mercaptoethanol. The sample, reduced to a suitable volume by ammonium sulphate precipitation, was applied to the column at a flow rate of 34 ml/h. Following

elution, with the equilibration buffer, fractions (6.8 ml) were collected and GST activity and absorbance at 280 nm were measured.

### 4.05 High pressure liquid chromatography (h.p.l.c)

This was performed at room temperature on a twin-pump Waters h.p.l.c. system (Waters Associates, Northwich, Cheshire, UK; see section 3.04b).

#### 4.05a Hydroxyapatite h.p.l.c.

Separation of human Pi- and Mu-class GST enzymes was achieved on a Bio-Gel HPHT h.p.l.c. grade hydroxyapatite column (Bio-Rad Laboratories, Hemel Hempstead, Herts, UK). Samples were first dialysed, against two changes, each of two litres, of 10 mM sodium phosphate buffer pH 7.0, containing 0.5 mM dithiothreitol, before application to the column at a flow rate of 0.5 ml/min. A linear gradient of 10-350 mM sodium phosphate buffer pH 7.0, containing 0.5 mM dithiothreitol and 0.4 mM $CaCl_2$ was employed to develop the column over 60 min. The $A_{280}$ of the eluate was monitored continuously and the GST activity was measured to allow identification of the isoenzyme-containing fractions.

#### 4.05b Reverse-phase h.p.l.c.

Reverse-phase h.p.l.c. on a μ Bondapak $C_{18}$ column (Millipore, Harrow, Middlesex, UK) was used to separate and isolate individual GST subunits, a method first described by Ostlund Farrants *et al* (1987). Samples, containing 0.1% (v/v) trifluoroacetic acid, were injected onto the column and the flow rate increased linearly, over 5 min, from 0.1 ml/min to 1.0 ml/min (see section 3.04b). The column was developed, over 60 min, with a linear 40-55% (v/v) acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid. The absorbance at 220 nm was monitored continuously to facilitate collection, by hand, of the protein containing peaks.

### 4.06 Fast protein liquid chromatography (f.p.l.c.)

This was performed, at room temperature, using a Pharmacia integrated f.p.l.c. system (Pharmacia, Laboratory Separation Division, Milton Keynes, Bucks., UK). The system comprised a model GP-250 gradient programmer, two model P-500 pumps, a UV-1 UV monitor, a pH monitor and a flow-through electrode.

4.06a Anion-exchange f.p.l.c.

Affinity purified human GST isoenzyme pools and a partially purified human Theta-class GST were further resolved on a Mono Q HR 5/5 column (Pharmacia) equilibrated with 20 mM Tris/HCl buffer pH 8.0, containing 0.5 mM dithiothreitol at a flow rate of 0.75 ml/min. Following extensive dialysis, at 4 °C, against the equilibration buffer, samples were applied to the column which was developed with a 0-1.0 M NaCl gradient in the same buffer. The gradient was established in two linear stages; 0-0.15 M NaCl in 60 min followed immediately by 0.15-1.0 M NaCl in either 10 or 25 min. Fractions (0.75 ml) were collected and analysed for protein concentration and GST activity.

4.06b Chromatofocusing f.p.l.c.

Chromatofocusing of human Mu-class GST isoenzymes, in the pH range 7-4, was performed on a Mono P HR 5/20 f.p.l.c. column (Pharmacia) pre-equilibrated in 25 mM Bistris/iminodiacetic acid buffer pH 7.1. Material to be subjected to chromatofocusing was first dialysed against two changes, each of 2 litres, of the same buffer before application to the column at a flow rate of 0.75 ml/h. The pH gradient was formed using Polybuffer 74 (diluted 1:30 (v/v) in water) adjusted to pH 4.0 with iminodiacetic acid, as the eluant. The pH was monitored continuously and the protein concentration and GST activity in the fractions (0.75 ml) collected were measured.

## 4.07 Purification of Mu-class isoenzymes from human skeletal muscle

Data on the purification and analysis of Mu-class isoenzymes from skeletal muscle tissue from three individuals (specimens 1,2 and 3; Table 4.01) will be presented. Cytosolic fractions from samples M1 (375 g), M2 (247 g) and M3 (354 g) were prepared separately (see section 4.02) in 20 mM Tris/HCl buffer pH 7.8, containing 200 mM NaCl and 0.5 mM dithiothreitol.

4.07a Affinity purification and resolution of GST isoenzyme forms

GST isoenzyme mixtures were isolated from the three skeletal muscle cytosolic fractions by affinity chromatography on columns of S-hexylglutathione-Sepharose 6B (see section 4.04a). Partial resolution of the affinity purified GST was then achieved by the use of anion-exchange chromatography, at pH 8.0, on a Mono Q HR 5/5 column with the

Pharmacia f.p.l.c. system (see section 4.06a). The samples were applied to the column in 5.0 ml portions (between 5-10 mg protein) which were then eluted separately at a flow rate of 0.75 ml/min. The column was developed in two stages with a linear salt gradient of 0-0.15 M NaCl over 60 min, followed immediately by 0.15-1.0 M NaCl over 10 min. Protein concentrations and GST activity, with CDNB as substrate, were measured in the fractions collected.

The chromatographic profiles obtained from Mono Q f.p.l.c. were different for each of the three individuals studied. However, a maximum of seven protein containing peaks (P1-P7) were resolved by this method. The protein containing fractions were pooled and analysed by SDS/PAGE before further purification. Three of the seven peaks (P1, P3 and P6) contained the major GST forms.

4.07b Purification of Mu-class isoenzymes

Anion-exchange on Mono Q f.p.l.c. of the affinity purified-material resulted in the isolation and purification of a Mu-class isoenzyme, which was present in peak P6 obtained from muscle samples M1 and M2. The other major GST forms, present in protein containing peaks P1 and P3, required further purification.

Peak P1 from skeletal muscle preparation M2 and peak P1 from skeletal muscle sample M3 each contained a different Mu-class enzyme. These GST were purified following chromatofocusing on Mono P f.p.l.c. in the pH range 7-4 (see section 4.06b). The Mu-class GST present in peak P1 from specimen M2 eluted from the chromatofocusing column at pH 5.9 and was recovered as peak P1-A, whereas the Mu-class GST present in peak P1 from specimen M3 eluted from the chromatofocusing column at pH 5.25 and was recovered as peak P1-A$^{/}$.

The material in peak P3, from all three skeletal muscle preparations (M1, M2 and M3), was subjected to chromatography on hydroxyapatite h.p.l.c. (see section 4.05a). A 10-350 mM sodium phosphate gradient resolved the GST activity present in this pool into two peaks, one containing a Pi-class enzyme (peak P3-A) and one containing a Mu-class enzyme (peak P3-B).

## 4.08 Purification of a Mu-class enzyme from human testicular tissue

A human testicular Mu-class GST was purified from testis tissue from one individual (specimen T3; see table 4.01).

A cytosolic fraction was prepared from 71 g of testicular tissue as described previously in section 4.02. Following dialysis, against two changes each of 5 litres of 20 mM Tris/HCl buffer, pH 7.8, containing 200 mM NaCl and 0.5 mM dithiothreitol, the cytosol was subjected to affinity chromatography on S-hexylglutathione-Sepharose 6B (see section 4.04a).

The affinity-purified GST pool was dialysed extensively against 20 mM Tris/HCl buffer pH 8.0, containing 0.5 mM dithiothreitol before being subjected to anion-exchange chromatography on a Mono Q HR 5/5 f.p.l.c. column (see section 4.06a). The GST isoenzyme mixture was applied to the column in 5.0 ml portions (containing 5-10 mg protein) which were then chromatographed separately. A two step salt gradient was used to develop the column, this involved a linear 0-0.15 M NaCl gradient, established over 60 min, followed immediately by a linear 0.15-1.0 M NaCl gradient over 25 min. Following measurement of protein concentrations and GST activity in the fractions collected those containing protein were pooled and analysed by SDS/PAGE.

The protein peak (Pvii) resolved by the second stage of the salt gradient, eluting at approx. 400 mM NaCl, was found to contain a Mu-class enzyme. The purification of this GST was, therefore, possible using two chromatographic steps.

## 4.09 Purification of human hepatic Mu-class enzymes

Hepatic Mu-class isoenzymes were purified from one individual (specimen L4; Table 4.01) and the isoenzyme profile in the skeletal muscle and cerebrum from the same individual were also analysed.

### 4.09a Affinity purification and separation of hepatic Mu-class GST

Cytosol was prepared from human liver tissue (411g), as described in section 4.02. A GST isoenzyme pool was isolated from the cytosolic fraction following affinity chromatography on two columns (1.6 cm x 20 cm) of S-hexylglutathione-Sepharose (see section

4.04a) which were run in parallel. Chromatography on a column (1.6 cm x 20 cm) of Bio-Rad HT grade hydroxyapatite was used to separate the GST isoenzymes present in the affinity-purified pool according to their class. This procedure is described in detail in section 4.04c. A linear gradient of 10-250 mM sodium phosphate buffer pH 7.0, containing 0.5 mM dithiothreitol, resolved the GST activity present into two peaks. Those fractions containing GST activity were combined into two pools. The GST activity which eluted in a peak between 80-140 mM sodium phosphate (PI) contained isoenzymes of the Mu-class and was, therefore, retained for further purification.

4.09b Resolution of multiple hepatic Mu-class isoenzymes

Conventional chromatofocusing, described in section 4.04d, in the pH range 8.5-5.5, was used to resolve the hepatic Mu-class isoenzymes expressed by this individual into three protein containing peaks each containing a distinct Mu-class enzyme.

4.09c Analysis of isoenzyme profiles in skeletal muscle and cerebrum

Skeletal muscle (240g; sample M4) and cerebrum (540 g; sample C4) were homogenized 1:4 in 20 mM Tris/HCl buffer pH 7.8, containing 200 mM NaCl and 0.5 mM dithiothreitol, and cytosols were prepared as described in section 4.02. Following affinity chromatography, on S-hexylglutathione-Sepharose 6B (see section 4.04a), the samples were subjected to anion-exchange chromatography, at pH 8.0, on a Mono Q column. The Mono Q column was developed with linear salt gradients established in two stages as described in section 4.06a. Chromatography of the sample purified from skeletal muscle involved the use of a 0-0.15 M NaCl gradient, developed over 60 min, followed immediately by a 0.15-1.0 M NaCl gradient over 10 min. By contrast, chromatography of the cerebrum sample involved the use of a 60 min 0-0.15 M NaCl gradient which was followed by a 0.15-1.0 M gradient established over 25 min. GST activity, with CDNB as substrate, and protein concentrations were measured in the fractions collected and those fractions containing protein were further analysed by SDS/PAGE.

## 4.10 Purification of a Theta-class GST from human liver

A human hepatic GST with high activity towards 1-menaphthyl sulphate (1-MS) and no apparent activity towards CDNB was purified from a liver sample from one individual.

Immediately following the preparation of a cytosolic fraction from 624 g of human liver (sample L5; Table 4.01), as described in section 4.02, the cytosol was applied to two columns (4.4 cm x 90 cm) of DE-52, equilibrated with 10 mM Tris/HCl buffer, pH 8.25, containing 1 mM EDTA and 0.5 mM dithiothreitol (see section 4.04e). The two columns, which were run in parallel, were washed with approx. one column volume of the equilibration buffer before each was developed with a 0-300 mM NaCl gradient in the same buffer. The fractions (11.5 ml) collected were analysed for GST activity towards CDNB and 1-MS as well as absorbance at 280 nm. A single peak of activity towards 1-MS eluted, between 110-160 mM NaCl on the gradient, from each of the columns.

Those fractions which contained activity towards 1-MS following DEAE-cellulose chromatography were pooled and applied immediately to a column (3.3 cm x 45 cm) of Bio-Rad HT grade hydroxyapatite equilibrated in 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.5 mM dithiothreitol (see section 4.04c). A 10-250 mM sodium phosphate gradient was used to develop the column and the eluate was monitored for enzyme activity. The fractions (4.7 ml) containing activity towards 1-MS, that eluted between 80-120 mM sodium phosphate, were pooled for further purification.

Dye-ligand chromatography on Matrex™ Gel Orange A was performed on the hydroxy-apatite purified material. The sample, which had been dialysed extensively against 10 mM sodium phosphate buffer pH 6.8, containing 1 mM EDTA and 0.5 mM dithiothreitol, was applied to a pre-equilibrated column (1.6 cm x 26 cm) as described in section 4.04b. Following the formation of a non-linear 0-1.0 M KCl the fractions collected were measured for GST activity.

The final stage in the purification involved anion-exchange chromatography on Mono Q f.p.l.c. (see section 4.06a). Those fractions which contained activity towards 1-MS, which had been retained by the Matrex™ Gel Orange A column, were pooled and dialysed extensively against 20 mM Tris/HCl buffer pH 8.0, containing 0.5 mM dithiothreitol, before application to Mono Q f.p.l.c. The sample was loaded onto the column in 5.0 ml portions (approx. 0.75 mg) which were each chromatographed separately. The gradient was formed in two linear stages consisting of 0-0.15 M NaCl formed over 60 min followed immediately by 0.15 -1.0 M NaCl formed over 10 min. Protein concentration, GST activity with 1-MS as substrate and GSH peroxidase activity with cumene hydroperoxide as substrate were measured in the fractions collected.

### 4.11 Purification of a Theta-class GST from mouse liver

A murine hepatic GST with activity towards 1-MS was purified from the livers of adult C57BL6 male mice.

Cytosol, from 134 g of mouse liver, was prepared in 10 mM Tris/HCl buffer pH 8.25, containing 1 mM EDTA and 2 mM 2-mercaptoethanol as described in section 4.02. Following dialysis, for 18 h at 4 °C, against two changes each of 5 litres of the same buffer, the sample was applied to a pre-equilibrated DE-52 column (4.4 cm x 90 cm) at a flow rate of 46 ml/h (see section 4.04e). A 0-300 mM NaCl gradient, in the running buffer, was used to develop the column. The fractions (11.5 ml) containing GST activity with the substrate 1-MS, which eluted between 20 mM and 60 mM NaCl on the gradient, were pooled for further purification.

Ammonium sulphate precipitation of protein between 30-90% saturation was performed on the material which was obtained following anion-exchange chromatography as described in section 4.03. The protein precipitated between 30-90% $(NH_4)_2SO_4$ saturation was resuspended in 10 mM sodium phosphate buffer pH 7.0, containing 1 mM EDTA and 2 mM 2-mercaptoethanol. Chromatography on a Sephadex G-100 gel filtration column (4.4 cm x 90 cm) was then used to further purify the Theta-class GST. See section 4.04f for details of this procedure.

The fractions obtained from the gel filtration column which contained GST activity were pooled prior to further chromatography on a column (1.6 cm x 30 cm) of Bio-Rad HT grade hydroxyapatite pre-equilibrated with 10 mM sodium phosphate buffer pH 7.0, containing 1 mM EDTA and 2 mM 2-mercaptoethanol (see section 4.04c). This column was developed with a 10-250 mM sodium phosphate gradient and the fractions collected were analysed for absorbance at 280 nm, GST activity with CDNB and 1-MS as substrates and glutathione peroxidase activity with cumene hydroperoxide as substrate. A single peak of GST activity with 1-MS as substrate eluted between 80-120 mM sodium phosphate. The final chromatographic step involved conventional chromatofocusing in the pH range 9.5-6.5 as described in detail in section 4.04d.

# PART III

# RESULTS

# Chapter 5 Purification of Mu-class GST from human skeletal muscle

## 5.01 Human skeletal muscle GST

The existence of a GST in skeletal muscle tissue, that was distinct from the hepatic GST, was first described by Laisney *et al.* (1984), who used starch gel electrophoresis to analyze GST activity in various human tissues. Subsequently, other workers (Suzuki *et al.*, 1987; Board *et al.*, 1988) purified a transferase expressed in skeletal muscle, called GST4, which is structurally related to the Mu evolutionary class. The reports of Laisney *et al.* (1984) and Board *et al.* (1988), however, present conflicting evidence as to the existence of heterodimeric Mu-class GST. Singh *et al.* (1988) have isolated several skeletal muscle GST; two of the minor forms, which were not observed in all of the samples examined, had sequence homology with the hepatic Mu-class enzymes. However, the major muscle isoenzyme described by these workers, which was present in all of the samples analysed, was distinct, both immunologically and catalytically, from any of the known Alpha-, Mu- or Pi-class GST.

In this thesis the skeletal muscle GST from a number of individuals have been purified in order to clarify the discrepancies which exist in the literature and to examine the possibility of inter-individual variation in the expression of these isoenzymes.

## 5.02 A purification strategy for skeletal muscle GST isoenzymes

Skeletal muscle GST have been isolated from a number of individuals. The results of the purification of GST from three of the tissue samples will be presented in detail in this chapter.

A combination of f.p.l.c and h.p.l.c. techniques were used to resolve affinity-purified GST isoenzymes. Initially, a partial separation of the GST forms present in S-hexylglutathione affinity-purified pools was achieved on Mono Q anion-exchange f.p.l.c. Further purification was carried out by either chromatofocusing on Mono P f.p.l.c. or hydroxyapatite h.p.l.c. These procedures allowed the isolation of four separate Mu-class GST and a Pi-class GST

from skeletal muscle. The steps involved in the purification of the Mu-class GST are described in detail in section 4.07 and summarized in Scheme 5.01.

## 5.03 Variation in the expression of GST isoenzymes in human skeletal muscle

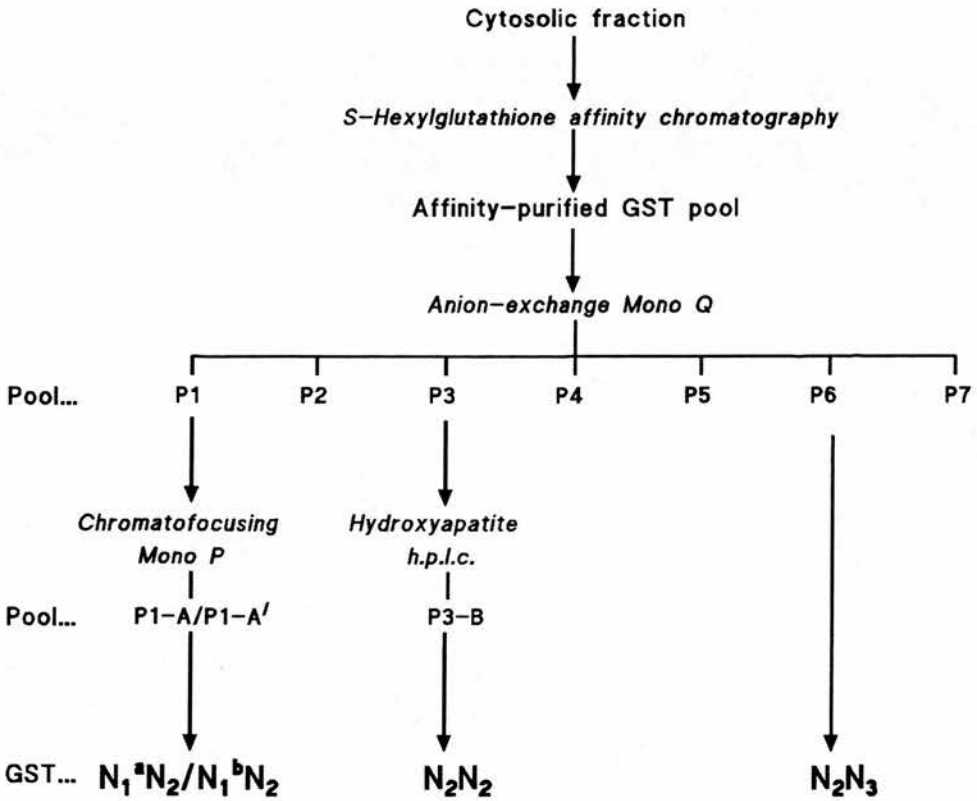
### 5.03a Analysis of GST isoenzyme pools from different individuals

S-Hexylglutathione affinity-purification of skeletal muscle cytosol from specimens M1, M2 and M3 resulted in isoenzyme mixtures which accounted for 0.22, 0.20 and 0.19% of the total cytosolic protein from each of the muscle samples respectively. When the isoenzyme profiles of two of these pools (from specimens M1 and M2) were examined by analytical isoelectric focusing (Figure 5.01) it was apparent that one band, of pI 5.65, was present in the pool obtained from skeletal muscle M2, whereas it was absent in the M1 pool. Isoelectric focusing also revealed the existence of two other predominant proteins, expressed by both individuals, which focused at pH 5.3 and pH 4.7.

### 5.03b Isolation of GST forms

Separation of the affinity-purified GST isoenzyme mixtures, from the seven skeletal muscle samples studied, on anion-exchange f.p.l.c. resulted in profiles which differed from specimen to specimen. The elution profiles obtained from samples M1, M2 and M3 are presented in Figure 5.02, together with SDS/PAGE analysis of the protein containing fractions.

Material from skeletal muscle M1 was resolved by Mono Q into seven protein-containing pools, two of which (P3 and P6) contained the major GST forms (Figure 5.02a). SDS/PAGE showed that peaks P1 and P2, which did not have GST activity towards 1-chloro-2,4-dinitrobenzene, both contained a single band with a subunit $M_r$ of 28500 (Figure 5.02b). P3 the first major peak, contained the 28500-$M_r$ polypeptide together with bands which co-migrated with the human placental 24800-$M_r$ GST $\pi$ standard and with a 26000-$M_r$ standard. The minor peaks P4 and P5 contained polypeptides of the same electrophoretic mobility as that of peak P3; however, P5 contained an additional band with an estimated subunit $M_r$ of 22500, which probably represents glyoxylase I (Hayes, 1988). The second major peak (P6) yielded two electrophoretic bands, one of which co-migrated




**Scheme 5.01 Purification of Mu-class GST from human skeletal muscle**

**Figure 5.01 Isoelectric focusing of affinity-purified GST pools from human ske muscle**

Isoelectric focusing of S-hexylglutathione-affinity-purified cytosolic GST from hu skeletal muscle samples M1 and M2 (20 $\mu$g of protein) was performed using a broad range gel (pH 3.5-9.5) in thin-layer 5% (w/v) polyacrylamide. The pI values of the pro calibration standards, which were run in the two outer tracts, are indicated.

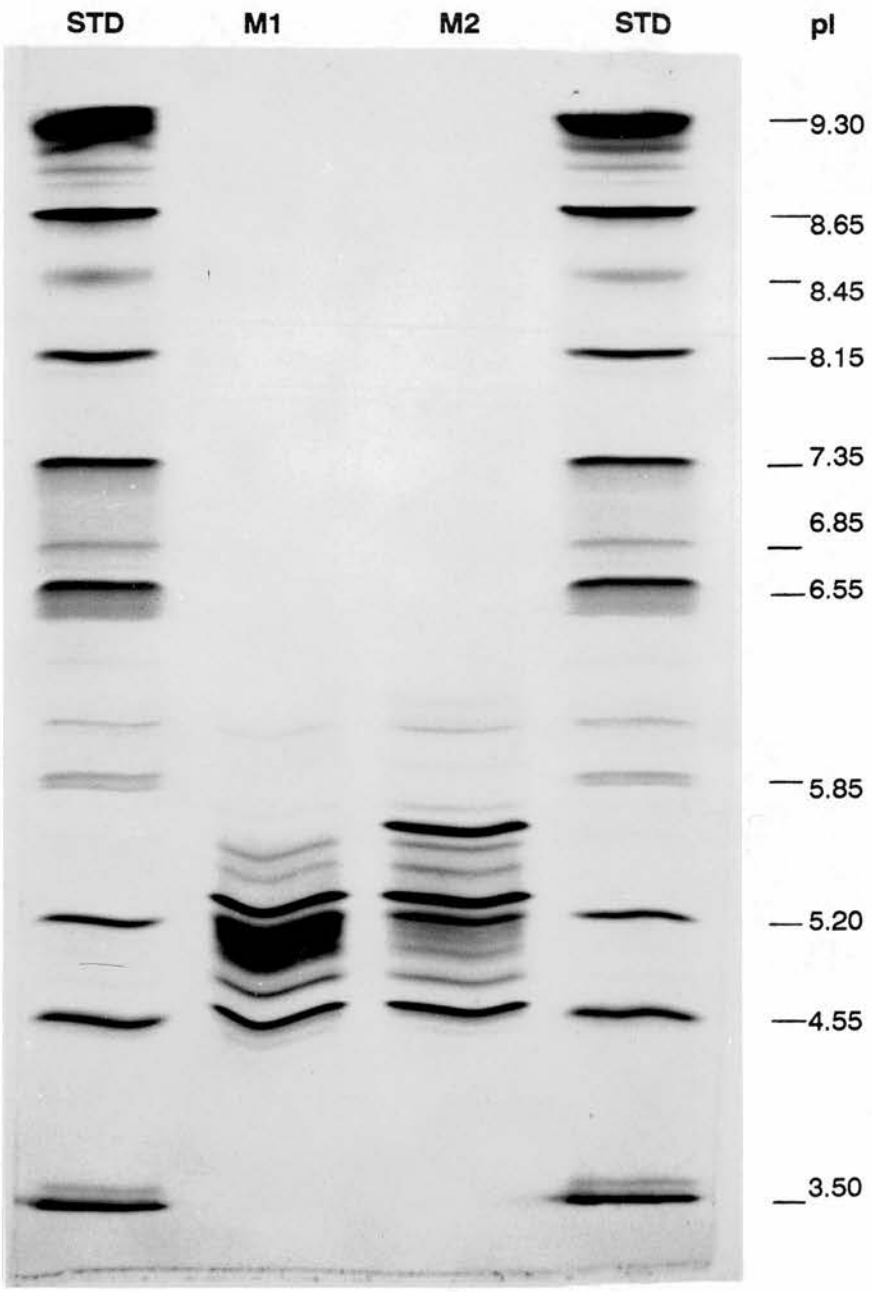
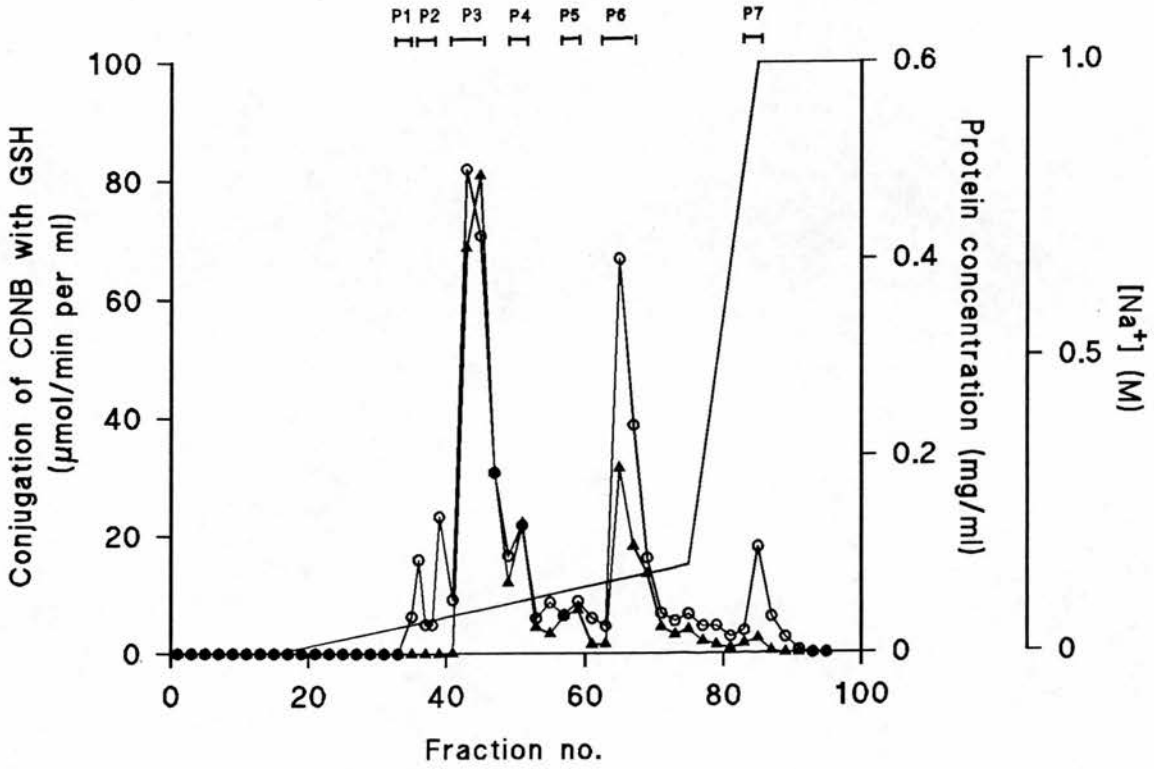



**Figure 5.01**

**Figure 5.02 Resolution of human skeletal muscle GST by anion-excha chromatography and SDS/PAGE analysis of individual peaks**

Anion-exchange f.p.l.c. on Mono Q of S-hexylglutathione-affinity-purified GST from hu skeletal muscle samples M1, M2 and M3 was performed as described in section 4. Panels a, c and e show the elution profiles obtained following chromatography of mat purified from specimens M1, M2 and M3 respectively. A 0-1.0 M NaCl gradient, in 20 Tris/HCl pH 8.0 containing 0.5 mM dithiothreitol, was established in two steps as sh by the straight lines. GST activity with 1-chloro-2,4-dinitrobenzene (▲) and pr concentrations (○) were measured. The protein containing fractions, from preparat M1, M2 and M3 were pooled, as indicated by the horizontal bars, and subjecte SDS/PAGE as shown in panels b, d and f respectively. The resolving gel contained (w/v) polyacrylamide and 0.32% (w/v) N,N′-methylenebisacrylamide. The positions o human GST markers $\mu$ ($M_r$ 26700) and $\pi$ ($M_r$ 24800), which were run in parallel, indicated. The first lane on each gel contained the total S-hexylglutathione-affinity-pur material (TOT; 6 $\mu$g of protein) from each muscle specimen. The following la contained the combined fractions from Mono Q (4 $\mu$g of protein) as indicated.

(a)




(b)

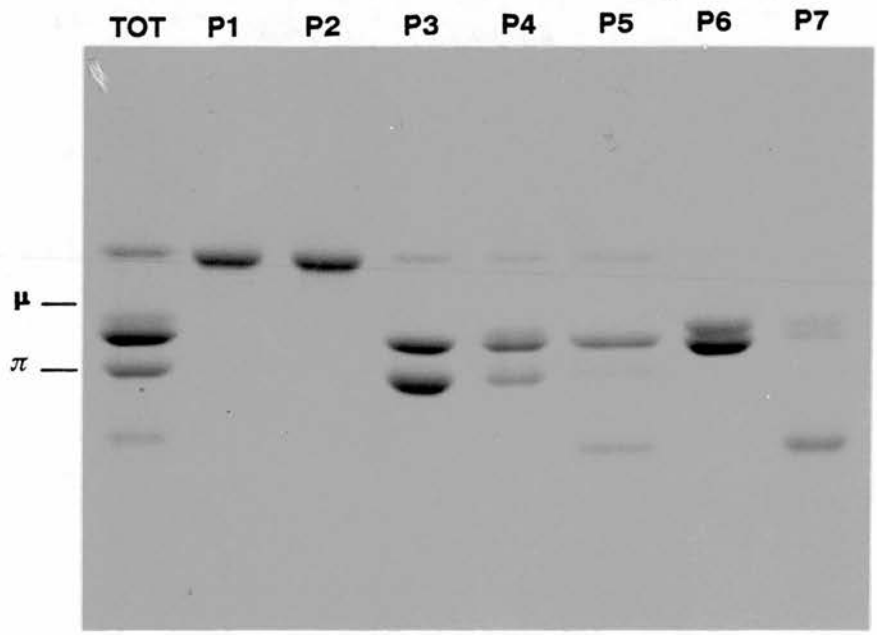
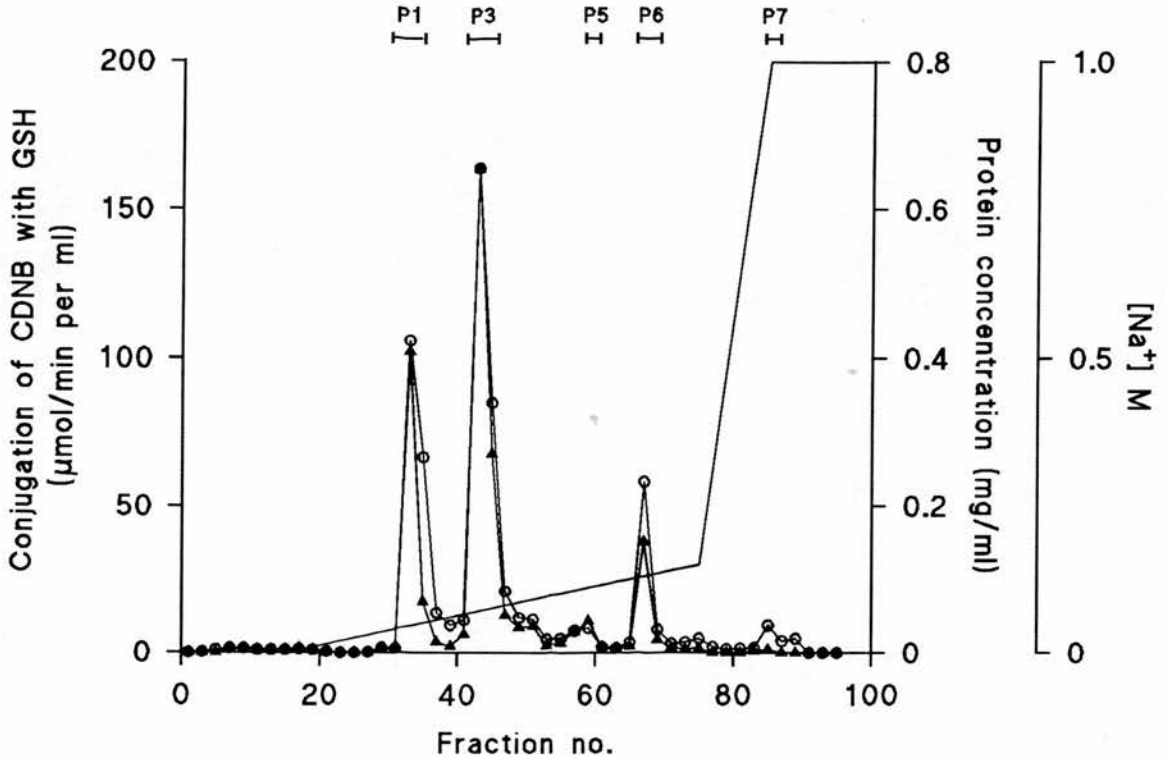



**Figure 5.02**

(c)




(d)

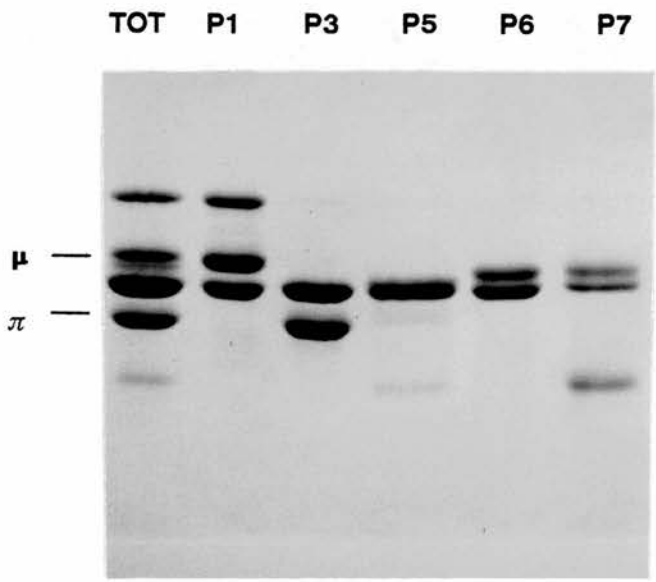
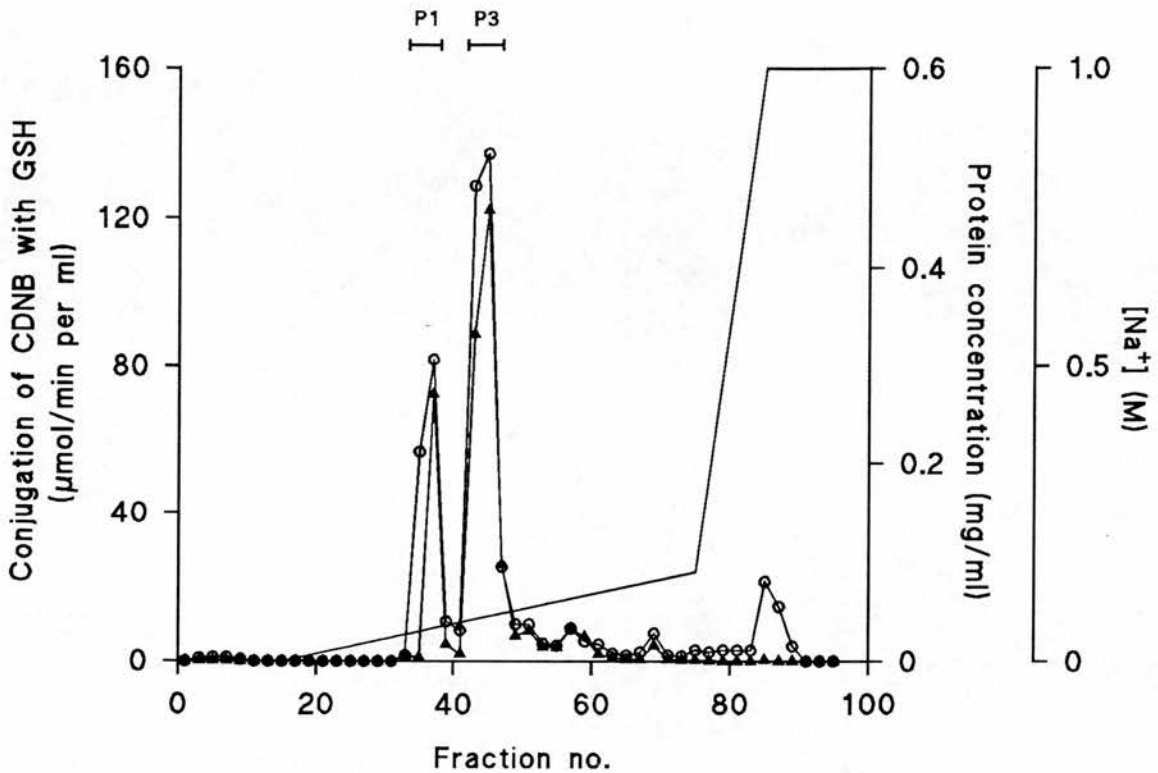



**Figure 5.02**

(e)




(f)

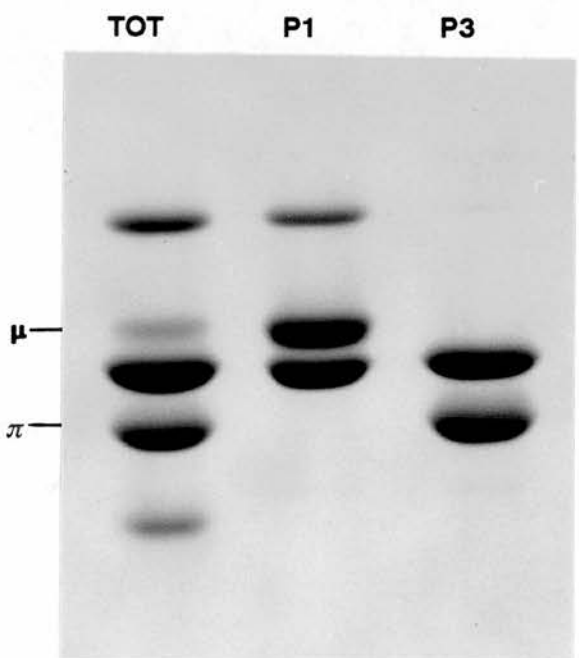



**Figure 5.02**

with the 26000-$M_r$ standard and a second band with a subunit $M_r$ of 26300. This 26300-$M_r$ band was also observed in peak P7, as was the 22500-$M_r$ polypeptide tentatively identified as glyoxylase I.

By contrast, when the affinity-purified GST pool from skeletal muscle sample M2 was subjected to the same anion-exchange chromatography step only five peaks were resolved by f.p.l.c. on Mono Q (Figure 5.02c). The most significant difference between the elution profile of specimen M2 and that of M1 was the increased size of the first peak, P1, and the fact that unlike P1 from M1, this pool contained GST activity towards 1-chloro-2,4-dinitrobenzene. When this peak from M2 was examined by SDS/PAGE (Figure 5.02d), three subunit bands were observed; the one with the slowest mobility was the 28500-$M_r$ polypeptide, the second band co-migrated with the human hepatic GST μ standard, of subunit $M_r$ 26700, and a third band co-migrated with the 26000-$M_r$ standard. Apart from the virtual absence of the 28500-$M_r$ polypeptide, peaks P3, P5, P6 and P7 appeared to have the same subunit compositions as the corresponding peaks on the elution profile obtained from sample M1.

The elution profile obtained following chromatography of the affinity-purified material from specimen M3 was similar to that of specimen M2. Five protein containing peaks were observed (Figure 5.02e), however, only two major GST forms were obtained and these were recovered in peaks P1 and P3. Electrophoretic analysis of peaks P1 and P3 (Figure 5.02f) revealed that they contained subunits with the same mobility as those present in peaks P1 and P3 from skeletal muscle M2.

Hence, specimen M1 expressed GST subunits of $M_r$ 24800, 26000 and 26300, whereas both specimens M2 and M3 expressed not only these three polypeptides but an additional GST subunit of $M_r$ 26700.

## 5.04 Purification of skeletal muscle GST

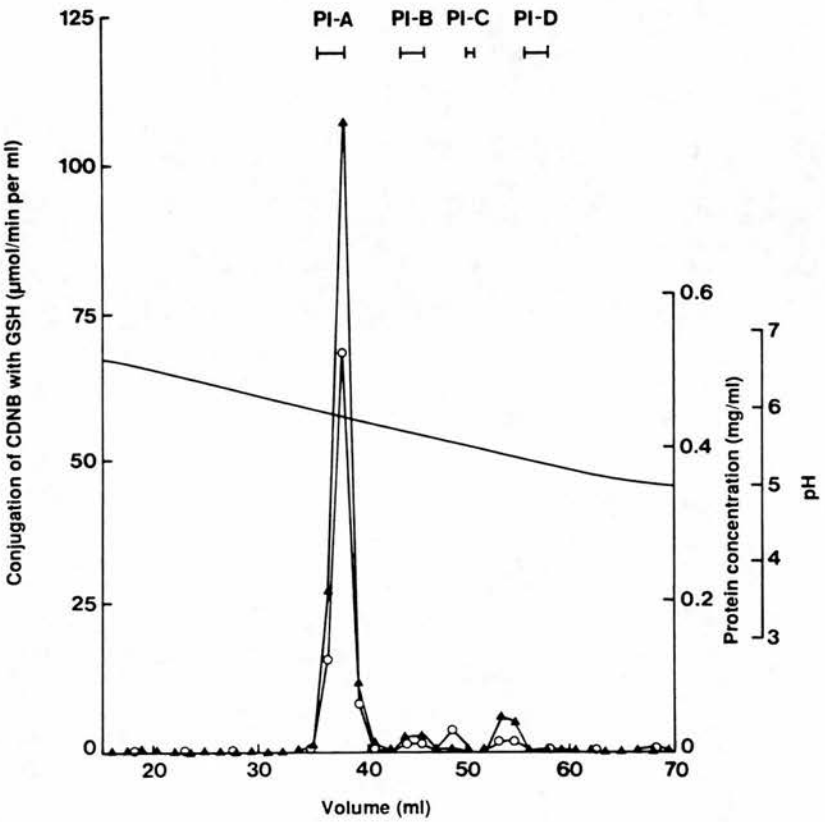
### 5.04a Resolution of muscle GST by chromatofocusing f.p.l.c.

The GST that were eluted from Mono Q in peak P1 were purified further by chromato-focusing on Mono P. The material recovered in peak P1 from skeletal muscle specimen M2 was resolved on this column into one major peak P1-A and three minor peaks, P1-B, P1-C and P1-D (Figure 5.03a). The protein-containing fractions were combined and their

**Figure 5.03 Chromatofocusing of partially purified skeletal muscle GST isoenzy and SDS/PAGE of resulting peaks**

Skeletal muscle GST activity, from specimens M2 and M3, purified by S-hexylglutath affinity chromatography and anion-exchange chromatography on Mono Q (P1), resolved by chromatofocusing on Mono P f.p.l.c. as described in section 4.06b (pa and panel c respectively). Transferase activity with 1-chloro-2,4-dinitrobenzen substrate (▲) and protein concentration (○) were measured, and the pH was monit (___). Fractions were combined, as indicated by the horizontal bars, and the su composition was analysed by SDS/PAGE (panel b and panel d). The first lane on gel was loaded with the material applied to the chromatofocusing column (P1; 4 $\mu$g) shown, the following lanes contained the pools obtained from Mono P; P1-A (4 $\mu$g), (1.5 $\mu$g), P1-C (4 $\mu$g) and P1-D (3 $\mu$g) from specimen M2 and P1-A′ (2.5 $\mu$g) specimen M3. The mobility of the marker protein human GST $\mu$ ($M_r$ 26700) which run in parallel, is indicated.

(a)




b)

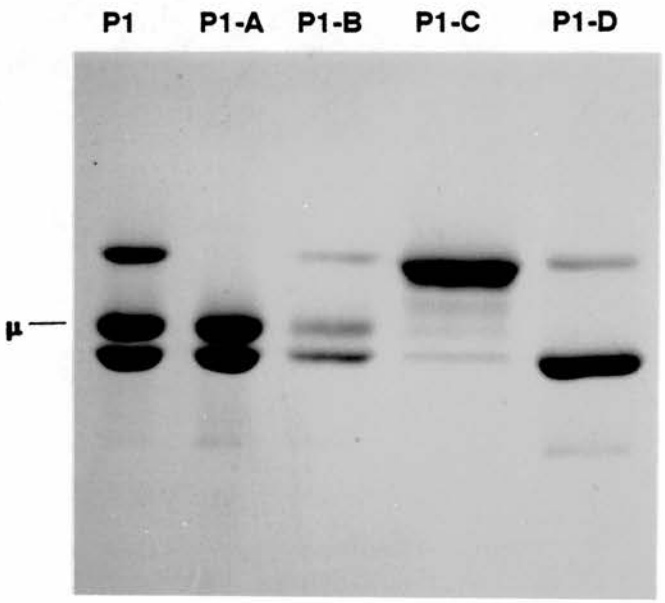
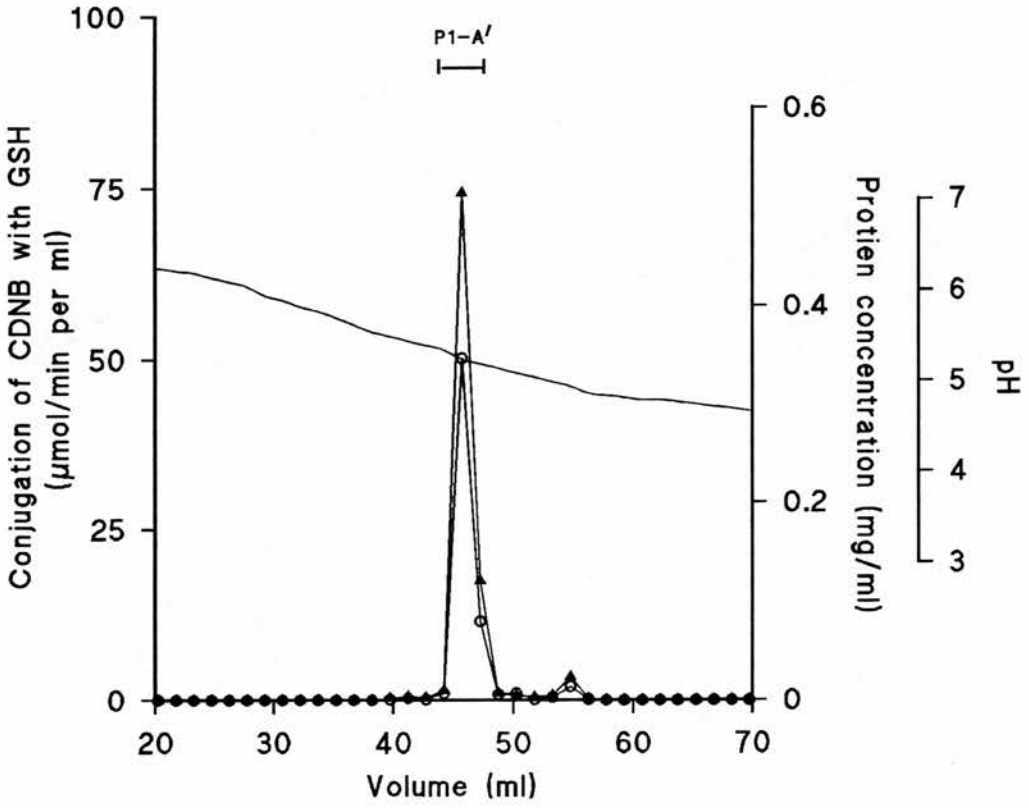



**Figure 5.03**

(c)




(d)

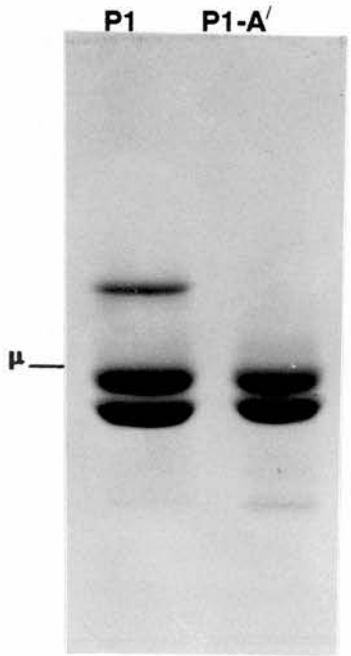



**Figure 5.03**

subunit compositions examined by SDS/PAGE (Figure 5.03b). Peak P1-A, which was eluted from the chromatofocusing column at pH 5.9, contained the two subunits that co-migrated during SDS/PAGE with the liver GST standards of $M_r$ 26000 and 26700. Electrophoresis showed that peak P1-B also contained these two subunits of $M_r$ 26000 and 26700 as well as the polypeptide of $M_r$ 28500. P1-C, the only peak which did not have GST activity with 1-chloro-2,4-dinitrobenzene as substrate, contained the contaminant polypeptide of $M_r$ 28500. Peak P1-D contained trace amounts of the contaminant and the GST subunit with $M_r$ 26000. When the GST present in peak P1 from skeletal muscle M3 were subjected to the same chromatofocusing step only one protein peak, which eluted at pH 5.25, was recovered from the column (Figure 5.03c). SDS/PAGE showed that this peak (P1-A′) also contained two subunits of $M_r$ 26000 and 26700 (Figure 5.03d).

5.04b Separation of a Mu-class and a Pi-class skeletal muscle GST

Hydroxyapatite h.p.l.c., a technique that resolves the different classes of human hepatic GST (Hussey *et al.*,1986), was employed to separate the subunit forms present in peak P3 obtained from all three muscle specimens. The profile obtained when material from specimen M2 was applied to this column is illustrated in Figure 5.04a. The first peak, which was eluted on the early part of the phosphate gradient, indicated the presence of Pi-class GST, and the second peak, which was eluted in the middle part of the gradient, indicated the presence of Mu-class subunits. Analysis by SDS/PAGE (Figure 5.04b) confirmed that the first peak (P3-A) contained the subunit with the same mobility as GST $\pi$, whereas the second peak (P3-B) contained the subunit with an estimated $M_r$ of 26000. Similar results were obtained when the GST present in peak P3 from skeletal muscle specimens M1 and M3 were separated by chromatography on hydroxyapatite h.p.l.c.

5.04c Characteristics of isoenzymes purified

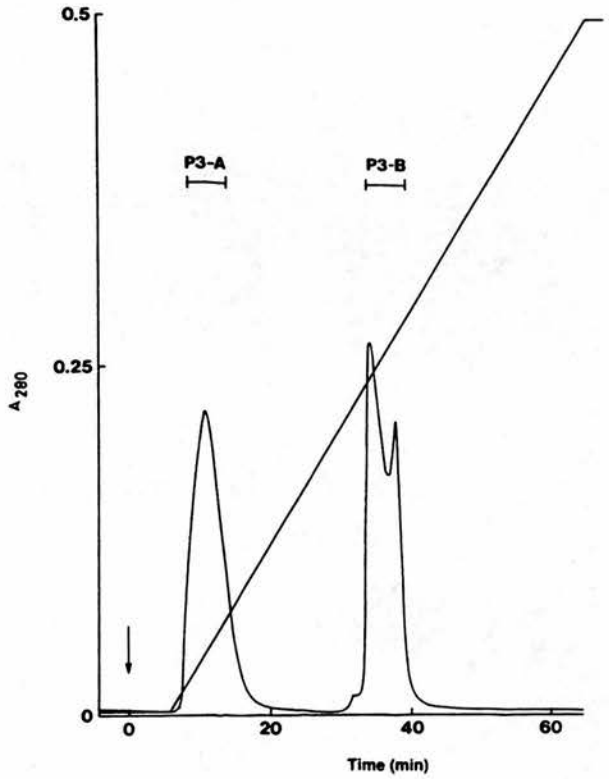
The results of the purification of GST from skeletal muscle specimens M1, M2 and M3 are summarized in Table 5.01. A different profile of GST isoenzymes was obtained from the skeletal muscle tissue of each of the three individuals studied.

Each of the muscle samples from the three individuals expressed two Mu-class isoenzymes and a Pi-class isoenzyme. The two Mu-class, which were designated GST $N_2N_3$ and GST $N_2N_2$, were recovered respectively in peak P6 from Mono Q, and in peak P3-B obtained following hydroxyapatite h.p.l.c. The GST $N_2N_3$ from specimen M3 was not

**Figure 5.04 Hydroxyapatite chromatography of partially purified skeletal m GST isoenzymes and SDS/PAGE analysis of pooled fractions**

Enzymes purified from skeletal muscle specimen M1 by S-hexylglutathione-a chromatography and anion-exchange chromatography on Mono Q (P3) were f resolved by chromatography on Bio-Gel HPHT as described in section 4.05a (par The column was developed with a linear 10-350 mM sodium phosphate gra indicated by the straight line, and the $A_{280}$ was monitored (___). SDS/PAGE analysis combined fractions, indicated by the horizontal bars, is shown in panel b. The mob of the human GST markers $\mu$ ($M_r$ 26700) and $\pi$ ($M_r$ 24800) are indicated. The firs contained the material applied to the hydroxyapatite h.p.l.c. column (P3; 4 $\mu$g). following lanes were loaded with the combined fractions, P3-A (4 $\mu$g) and P3-B ( as shown.

(a)




(b)

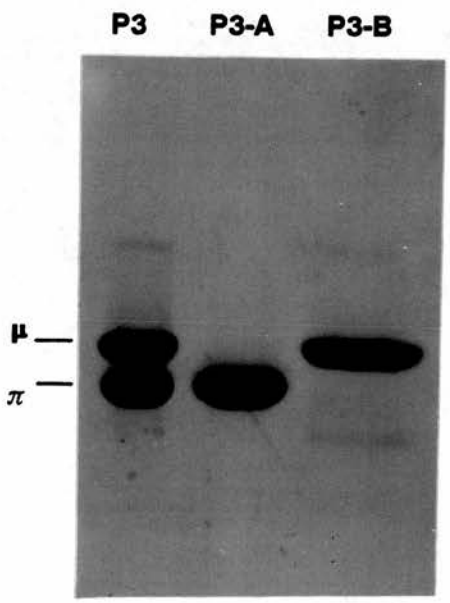



**Figure 5.04**

**Table 5.01 Purification of human GST isoenzymes from three skeletal muscle samples**

For experimental details see section 4.07. GST activity was measured with 1-chloro-2,4-dinitrobenzene at 37 °C.

| Fraction | Total protein (mg) | | | Total activity (μmol/min) | | | Specific activity (μmol/min per mg) | | | Recovery (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M1 | M2 | M3 | M1 | M2 | M3 | M1 | M2 | M3 | M1 | M2 | M3 |
| Cytosol | 9007 | 9742 | 12533 | 5419 | 4817 | 6063 | 0.60 | 0.49 | 0.48 | 100 | 100 | 100 |
| Affinity-chromatography pool | 19.8 | 19.4 | 23.3 | 2245 | 3817 | 4746 | 77.8 | 196.7 | 203.7 | 41.4 | 79.2 | 78.3 |
| Mono Q f.p.l.c. pools: | | | | | | | | | | | | |
| P1 | - | 3.1 | 1.3 | - | 631.2 | 361.4 | - | 201.0 | 282.3 | - | 13.1 | 6.0 |
| P3 | 3.9 | 4.1 | 7.2 | 933.0 | 1160 | 2108 | 240.5 | 283.6 | 292.8 | 17.2 | 24.1 | 34.8 |
| P6 ($N_2N_3$) | 2.7 | 0.9 | - | 253.0 | 1630 | - | 92.3 | 187.4 | - | 4.7 | 3.4 | - |
| Mono P f.p.l.c. pool: | | | | | | | | | | | | |
| P1-A ($N_1{}^aN_2$) | - | 1.12 | - | - | 152.0 | - | - | 135.7 | - | - | 3.2 | - |
| P1-A′ ($N_1{}^bN_2$) | - | - | 1.09 | - | - | 221.8 | - | - | 203.5 | - | - | 3.7 |
| HPHT h.p.l.c. pools: | | | | | | | | | | | | |
| P3-A (Pi) | 0.66 | 0.73 | 1.44 | 74.0 | 87.6 | 149.3 | 112.5 | 120.0 | 103.7 | 1.4 | 1.8 | 2.4 |
| P3-B ($N_2N_2$) | 0.59 | 1.14 | 1.75 | 100.8 | 243.0 | 486.5 | 171.1 | 213.1 | 278.0 | 1.9 | 5.0 | 8.0 |

purified due to the very low level of its expression in this tissue, as indicated by the size of peak P6 on Mono Q. The Pi-class GST was resolved on hydroxyapatite h.p.l.c. in peak P3-A.

Specimens M2 and M3, however, each expressed a different, additional Mu-class GST to those described above which was isolated, in each case, using chromatofocusing f.p.l.c. GST $N_1{}^aN_2$ was recovered from specimen M2 in peak P1-A, which eluted at pH 5.9 from Mono P, and GST $N_1{}^bN_2$ was purified from specimen M3 as peak P1-A′ which eluted from the chromatofocusing column at pH 5.25.

The characterization of the Mu-class GST $N_1{}^aN_2$, $N_1{}^bN_2$, $N_2N_2$, and $N_2N_3$ will be presented in detail in Chapter 8.

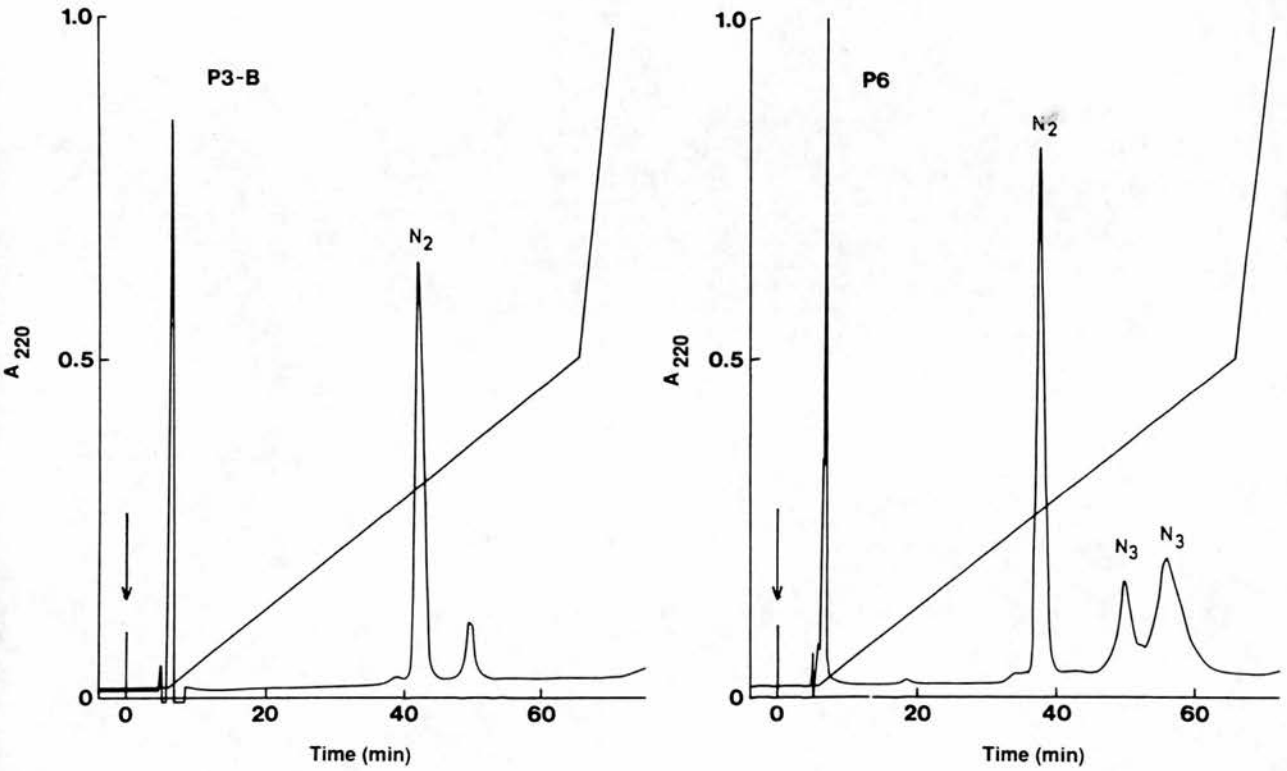
### 5.05 Isolation of skeletal muscle Mu-class GST subunits

Reverse-phase h.p.l.c. was used in an attempt to prepare separately the individual subunits present in the skeletal muscle GST isoenzymes (section 4.05b). This technique successfully resolved the $N_2$ and $N_3$ subunit and could also be used to purify further GST $N_2N_2$ (P3-B) by removing small quantities of the 28500-$M_r$ polypeptide that contaminated certain preparations. Figure 5.05a shows the elution profiles obtained when GST $N_2N_2$ (P3-B) and GST $N_2N_3$ (P6) , purified from skeletal muscle specimen M1, were applied to the μ Bondapak column. Electrophoretic analysis (Figure 5.05b) revealed that the major peak, which was present on both profiles eluting between 37 and 41 min, contained the $N_2$ subunit of $M_r$ 26000. The $N_3$ subunit, of $M_r$ 26300, was recovered in two peaks which eluted at 50 min and 56 min. The $N_1{}^a$ and $N_2$ subunits present in GST $N_1{}^aN_2$, isolated from skeletal muscle sample M2, were not resolved by this technique; both subunits eluted as a single peak at 40 min.

**Figure 5.05 Preparative reverse-phase h.p.l.c. of human skeletal muscle subunits and SDS/PAGE analysis of resulting peaks**

The GST pools P3-B, containing isoenzyme $N_2N_2$, and P6, containing isoenzyme from skeletal muscle sample M1 were each subjected to reverse-phase h.p.l.c. (pa on a Waters μ Bondapak $C_{18}$ column (10 $\mu$m particle size; 0.39 cm x 30 cm). The c was developed with a 40-70% (v/v) acetonitrile gradient in aq. 0.1% (v/v) trifluoro acid in two stages as shown by the straight lines. The $A_{220}$ of the eluate was mon continuously (___). The GST $N_2$ and $N_3$ subunits which were resolved by this method collected and freeze-dried prior to analysis by SDS/PAGE (panel b). The first track s contained P3, the GST pool obtained after anion-exchange chromatography on M of total S-hexylglutathione affinity purified material (4 $\mu$g). The second track cont pool P3-B purified on hydroxyapatite h.p.l.c. (4 $\mu$g). The $N_2$ subunit, purified from P3-B on reverse-phase h.p.l.c., was loaded into the third track shown. The fourth contained pool P6, purified by affinity chromatography and anion-exchange chroma phy on Mono Q (4 $\mu$g). The $N_2$ and $N_3$ subunits resolved by reverse-phase h.p.l.c pool P6 were loaded into the final three tracts as shown. The mobilities of the subunit markers $B_1B_2$ ($M_r$ 26000) and $\pi$ ($M_r$ 24800), which were run in paralle indicated.

(a)




(b)

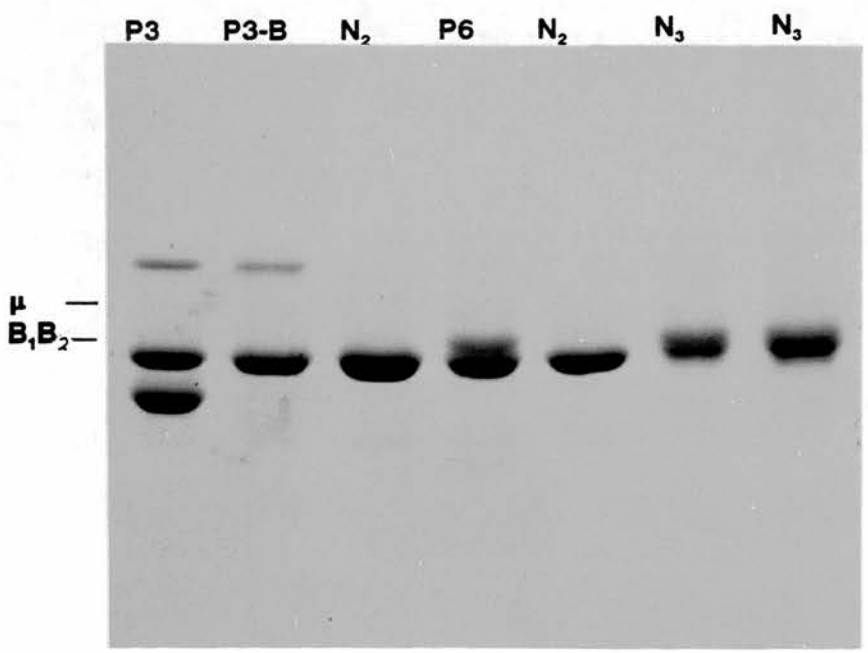



**Figure 5.05**

# Chapter 6 Purification of human testicular GST

## 6.01 Human testis GST

High concentrations of Basic (Alpha-class) and Near-neutral (Mu-class) GST have been found in human testis using radial immunodiffusion (Corrigall and Kirsch, 1988). Others (Aceto *et al.*, 1989), who attempted to characterize the GST isoenzymes present in testicular tissue, described preparations that they were unable to related to any of the GST characterized previously.

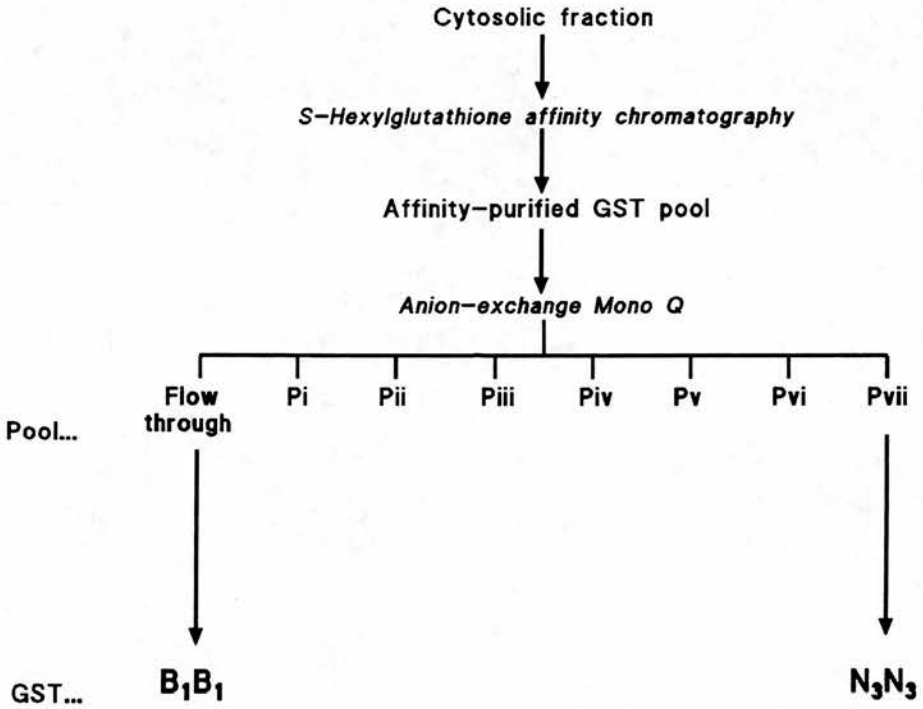
The purification of Alpha-class GST $B_1B_1$ and a distinct Mu-class GST from a human testis sample from one individual is presented in this chapter.

## 6.02 Purification protocol for human testis GST

To allow comparisons between the GST isoenzyme profile of human testis and that of skeletal muscle an identical purification strategy, to that described in the previous chapter, was used. Anion-exchange f.p.l.c. of an affinity-purified isoenzyme mixture resulted in the purification, in two chromatographic steps, of an Alpha-class and a Mu-class GST. Scheme 6.01 outlines the stages involved in the isolation of human testis GST; a more detailed account is given in section 4.08.

## 6.03 Resolution of testicular GST isoenzymes

Human testis cytosol was found to be a comparatively rich source of GST. The S-hexylglutathione-affinity-purified isoenzyme pool from specimen T3 accounted for 0.7% of the total cytosolic protein. When this isoenzyme mixture was subjected to anion-exchange f.p.l.c. 62.8% of the affinity-purified protein failed to be retained by the Mono Q column. The remainder of the affinity-purified pool was resolved on the salt gradient into seven peaks, P(i)-P(vii), all of which contained activity towards 1-chloro-2,4-dinitrobenzene (Figure 6.01a). In terms of protein recovered, however, the major GST forms were present in those fractions which passed straight through the column and in peaks P(iv) and P(vii).

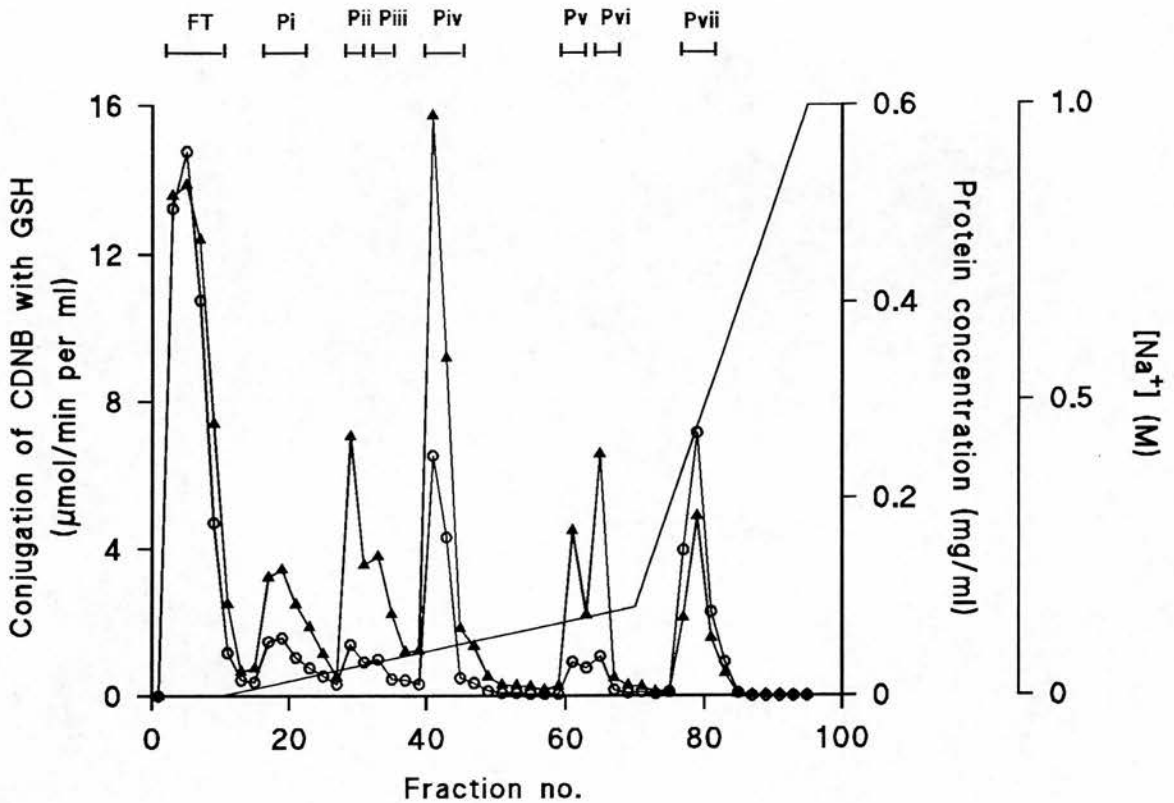



**Scheme 6.01 Purification of human testicular GST**

**Figure 6.01 Anion-exchange f.p.l.c. of affinity-purified GST isoenzymes from hu testis and SDS/PAGE of combined fractions**

S-Hexylglutathione-affinity-purified GST activity from human testis sample T3 was reso by anion-exchange chromatography on Mono Q (panel a) as described in section 4. The column was developed with a two-step linear 0-1.0 M NaCl gradient as shown b straight lines and the resulting fractions were assayed for protein concentration (○) GST activity with 1-chloro-2,4-dinitrobenzene as a substrate (▲). Those frac containing GST activity were pooled, as indicated by the horizontal bars, and subje to electrophoretic analysis (panel b). The first track on the SDS/PAGE gel shows the S-hexylglutathione-affinity-purified material (TOT; 6 $\mu$g). The second track was loa with the GST activity which was not retained by the Mono Q column (FT; 6 $\mu$g), and following seven tracts contained the peaks of activity resolved by the salt gradient (P*i*- 4 $\mu$g). The positions of the human GST subunit markers $\mu$ ($M_r$ 26700) and $\pi$ ($M_r$ 24 which were run in parallel, are indicated.

(a)




(b)

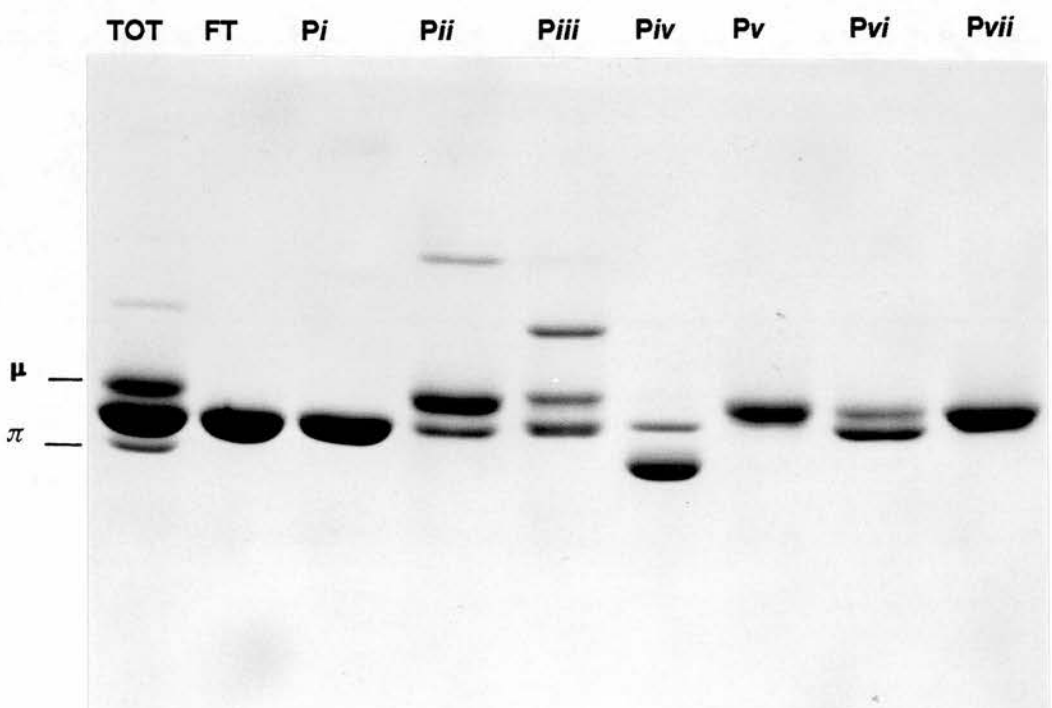



**Figure 6.01**

Electrophoretic analysis (Figure 6.01b) of the fractions eluting from the anion-exchange column revealed the presence of a single 26000-$M_r$ polypeptide in the flow-through material. The first peak, P(i), resolved on the gradient also contained a single band of subunit $M_r$ 26000. Peaks P(ii) and P(iii) contained polypeptides which co-migrated with the hepatic subunit standards of $M_r$ 26000 and 26700, but also contained additional high molecular weight subunits of $M_r$ 31500, in the case of P(ii), and 28500 in the case of P(iii). SDS/PAGE of peak P(vi) showed the presence of two bands; the predominant subunit co-migrated with the GST $\pi$ standard ($M_r$ 24800) and a second subunit which had an $M_r$ of 26000. Although the minor peaks P(v) and P(vi) were not well resolved they each appeared to have a different subunit composition; a single polypeptide of $M_r$ 26500 was present in P(v), whereas P(vi) contained bands of $M_r$ 26000 and 26300.

At least five cytosolic GST subunits ($M_r$ 26000, 26700, 24800, 26500 and 26300) were, therefore, expressed in testis sample T3. Details of the purification of the testicular GST enzymes are presented in Table 6.01.

## 6.04 Identification of human testis GST

### 6.04a Comparison of testicular GST with skeletal muscle isoenzymes

If one compares the elution positions of the testis GST subunits, following Mono Q, together with their subunit compositions, as determined by SDS/PAGE, with those resulting from Mono Q of affinity purified pools from skeletal muscle (see Figure 5.02), it is possible to make a tentative identification of some of the GST forms present. The major testis GST, however, which was not retained by the Mono Q column and possessed a subunit $M_r$ of 26000 (FT), was not present in any of the skeletal muscle samples analysed. A further electrophoretic characterization of this isoenzyme is presented in the following section.

The subunit size and elution position of testis peaks P(ii) and P(iii) indicate the presence of $N_1$ ($M_r$ 26700) and $N_2$ ($M_r$ 26000) subunits; peak P(iv), which eluted in the same position from Mono Q as peak P3 from skeletal muscle (60-75 mM NaCl), appears to contain a Pi-class enzyme ($M_r$ 24800) together with GST $N_2N_2$ ($M_r$ 26000); peak P(vi), represented by peak P6 from skeletal muscle, contains GST $N_2N_3$; and peak P(vii) contained a major testis Mu-class GST $N_3N_3$ ($M_r$ 26300). Details on the characterization of GST $N_3N_3$ are included in chapter 8. Unfortunately, due to paucity of material it was not possible to further

**Table 6.01 Purification of human testis GST isoenzymes**

For experimental details see section 4.08. GST activity was measured with 1-chloro-2,4-dinitrobenzene at 37 °C.

| Fraction | Total protein (mg) | Total activity ($\mu$mol/min) | Specific activity ($\mu$mol/min per mg) | Recovery (%) |
|---|---|---|---|---|
| Cytosol | 1886.8 | 1593.4 ± 4.9 | 0.84 ± 0.003 | 100 |
| Affinity-chromatography pool | 13.78 | 833.9 ± 9.9 | 60.5 ± 0.72 | 52.3 |
| Mono Q f.p.l.c. pools: | | | | |
| Flow-through ($B_1B_1$) | 5.43 | 296.1 ± 4.1 | 54.5 ± 0.8 | 18.6 |
| P(*i*) | 0.39 | 22.0 ± 0.1 | 56.4 ± 0.3 | 1.4 |
| P(*ii*) | 0.11 | 17.4 ± 0.1 | 158.2 ± 0.9 | 1.1 |
| P(*iii*) | 0.08 | 7.5 ± 0.15 | 93.8 ± 1.9 | 0.5 |
| P(*iv*) | 1.20 | 129.9 ± 0.7 | 108.2 ± 0.6 | 8.1 |
| P(*v*) | 0.07 | 6.8 ± 0.2 | 97.1 ± 2.8 | 0.4 |
| P(*vi*) | 0.09 | 18.0 ± 0.1 | 200.0 ± 1.1 | 1.1 |
| P(*vii*) ($N_3N_3$) | 1.28 | 19.39 ± 0.17 | 15.1 ± 0.1 | 1.2 |

characterize the GST subunits of $M_r$ 26000 and 26500 present in peaks P(i) and P(v) respectively.

### 6.04b Isoelectric focusing of testis Alpha-class GST

Analytical isoelectric focusing of a portion of the affinity purified isoenzyme mixture from testis sample T3 revealed the presence of two bands which focused in the basic region of the gel, at pI's of 8.9 and 8.73, and three minor bands in the acidic region at pI's of 5.25, 5.1 and 4.9 (Figure 6.02). The Mono Q flow-through material, which was analysed at the same time, contained the GST which focused at a pI of 8.9. The pI and subunit $M_r$ of this testis GST are identical to those of the hepatic GST $B_1B_1$ described by Stockman *et al.* (1985). The second basic band of pI 8.73, visible in the affinity purified pool, probably represents GST $B_1B_2$; this isoenzyme has been described as having a pI of 8.75 (Stockman *et al.*, 1985). If this is the case it is possible that peak P(i), resolved on Mono Q, which has the same subunit size as the human hepatic Alpha-class GST, also represents $B_1B_2$.

**Figure 6.02 Isoelectric focusing of human testicular GST isoenzymes**

GST pools obtained by S-hexylglutathione-affinity chromatography of a human t
cytosolic fraction (TOT; 20 $\mu$g), followed by anion-exchange chromatography on Mo
f.p.l.c. (FT; 5 $\mu$g) were each analysed by isoelectric focusing. The electrophoresis
carried out using a broad range gel (pH 3.5-9.5) in thin-layer 5% (w/v) polyacrylar
The first track contained protein pI calibration standards, the pI values of which
indicated.

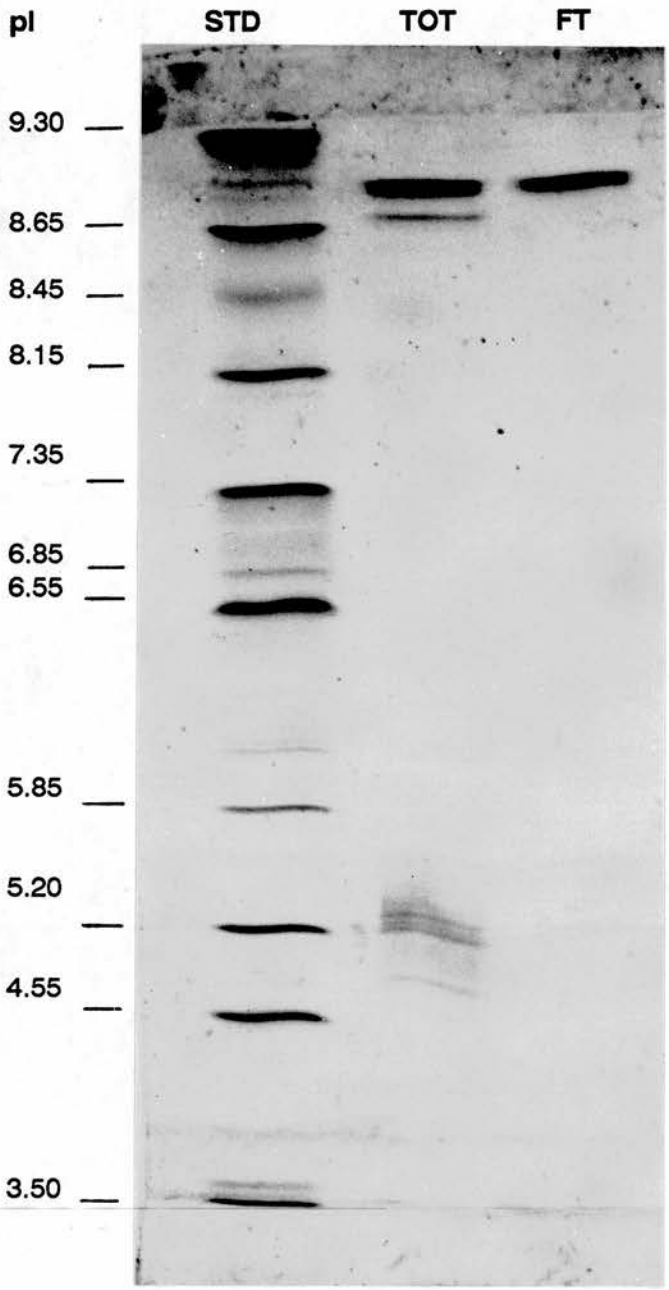



**Figure 6.02**

# Chapter 7 Purification of the allelic variants of the human hepatic Mu-class GST

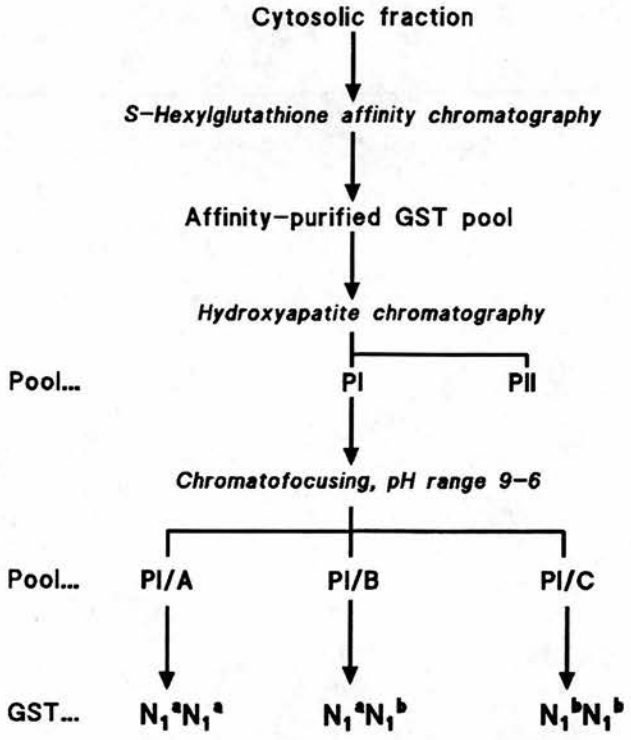
## 7.01 Human liver Mu-class GST

The Mu-class GST of human liver are represented by two well characterized isoenzymes termed μ (Warholm *et al.*, 1983) and ψ (Hussey *et al.*, 1986; Singh *et al.*, 1987a; Hayes, 1989). These homodimeric proteins are highly homologous (Hayes, 1989), and it has been suggested that they represent allelic variants coded at a single locus (Board, 1981; Strange *et al.*, 1984). The heterodimeric form of these enzymes was purified recently (Van Ommen *et al.*,1990), however the characterization of this GST was not described in detail. Between 40 and 45% of individuals fail to express either of these Mu-class GST (Strange *et al.*, 1984; Hussey *et al.*, 1987) and there is evidence to suggest that the hepatic Mu-class GST may provide a susceptibility marker for chemical carcinogenesis (Seidegard *et al.*, 1986, 1990)). A third hepatic Mu-class GST was identified by Stockman and Hayes, (1987); this homodimeric enzyme, called φ, was found in only one of 20 human livers examined.

## 7.02 Strategy for the analysis of $N_1$-type subunits

In this thesis the subunits which comprise GST μ and ψ have been designated $N_1^a$ and $N_1^b$ respectively. The purification of two homodimeric and one heterodimeric Mu-class GST from the liver of an individual (specimen L4; see Table 4.01) who expressed both $N_1^a$ and $N_1^b$ subunits is presented in this chapter. The abundance of GST in human liver cytosol permitted the isolation of these isoenzymes using conventional chromatographic procedures; affinity-purified material was resolved by hydroxyapatite chromatography followed by chromatofocusing in the pH range 9-6. The purification steps involved are described both in detail in section 4.09, and summarized in Scheme 7.01.

In an attempt to further establish the relationship between the $N_1$-type subunits and the $N_2$ and $N_3$ subunits, described in the preceding chapters, the GST present in skeletal muscle and cerebrum from the individual who expressed the two kinds of $N_1$ subunit were examined. The isoenzyme profiles of these tissues were analysed by subjecting affinity-




**Scheme 7.01 Purification of Mu-class GST from human liver**

purified material to anion-exchange f.p.l.c. and performing SDS/PAGE on the protein containing fractions.

### 7.03 Purification of three hepatic Mu-class GST

The GST isoenzyme mixture, isolated by S-hexylglutathione affinity-chromatography of cytosol from human liver specimen L4, accounted for 1.1% of the total cytosolic protein. Hydroxyapatite chromatography was used to separate the GST forms present in the affinity-purified material into two pools; one of which contained Alpha-class enzymes and a second which contained Mu-class GST. These pools were separately recovered as two protein peaks, named PI and PII, which eluted from the hydroxyapatite column between 50-65 mM and 100-125 mM sodium phosphate respectively (Figure 7.01a). Electrophoretic analysis of the fractions (Figure 7.01b) confirmed that peak PI contained a subunit which co-migrated with the hepatic GST $\mu$ standard ($M_r$ 26700), together with a minor 28500-$M_r$ contaminant, whereas PII contained a single band of $M_r$ 26000, which co-migrated with the human hepatic Alpha-class standard GST $B_1B_2$.

The hepatic Mu-class GST from specimen L4 (peak PI) were resolved by chromatofocusing into three protein-containing peaks, PI/A, PI/B and PI/C, which eluted from the column at pH 7.75, 7.45 and 7.15 respectively (Figure 7.02). Each of these peaks contained a distinct Mu-class GST; PI/A represented $N_1^aN_1^a$ (or $\mu$), PI/B represented $N_1^aN_1^b$ (a heterodimer composed of subunits from $\mu$ and $\psi$), and PI/C represented $N_1^bN_1^b$ (or $\psi$). Table 7.01 shows details on the purification of these hepatic Mu-class GST.

The electrophoretic, immunological and enzymatic characterization of the hepatic Mu-class isoenzymes in comparison with the Mu-class GST isolated from skeletal muscle (Chapter 5) and testis (Chapter 6) are presented in Chapter 8.

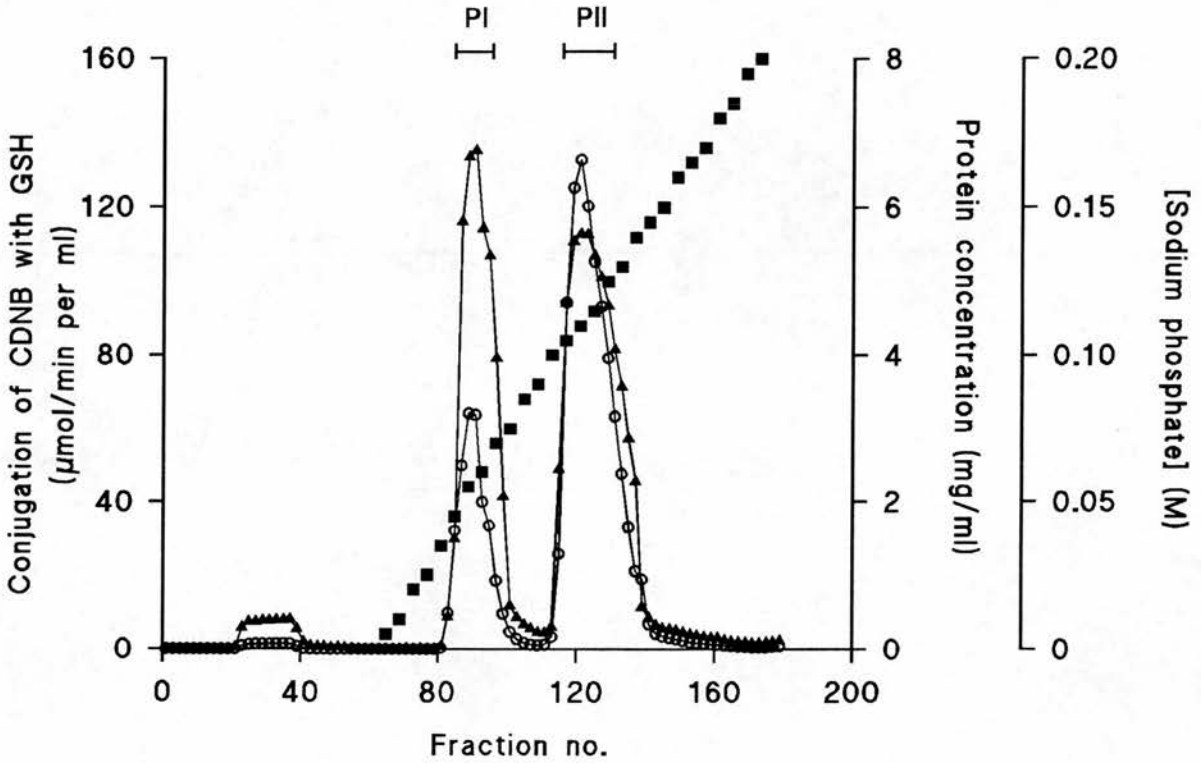
### 7.04 Expression of $N_1$-type subunits in extra-hepatic tissue

An affinity-purified pool from the skeletal muscle of the individual who expressed both the Mu-class GST $N_1^a$ and $N_1^b$ subunits was resolved by anion-exchange f.p.l.c. into six protein-containing peaks (Figure 7.03a). Subunit analysis of these peaks was achieved using SDS/PAGE (Figure 7.03b); peaks 1 and 2, which were poorly resolved, each

**Figure 7.01 Hydroxyapatite chromatography of affinity-purified GST isoenz from human liver and SDS/PAGE analysis of pooled fractions**

Affinity-purified GST isoenzymes from a human liver cytosolic fraction (specimen L4) subject to chromatography on a column of Bio-Rad HT grade hydroxyapatite as desc in section 4.04c (panel a). The column was developed with a linear 10-250 mM so phosphate gradient (▪), and GST activity, with 1-chloro-2,4-dinitrobenzene as sub (▲), and protein concentrations (○) were measured in each of the fractions collected two peaks of activity (PI and PII), resolved by the sodium phosphate gradient, pooled, as indicated by the horizontal bars, and analysed by SDS/PAGE (panel b) first track shown contained the total S-hexylglutathione-affinity-purified material (T $\mu$g) and the following two tracks were loaded with the pooled fractions (PI and PII; from hydroxyapatite chromatography. The mobilities of the human GST subunit ma $\mu$ ($M_r$ 26700) and $B_1B_2$ ($M_r$ 26000) are indicated.

(a)




(b)

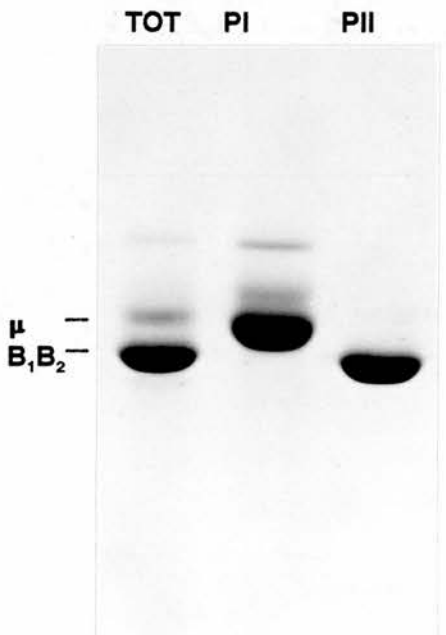



**Figure 7.01**

**Figure 7.02 Resolution of human hepatic Mu-class GST by chromatofocusi**

Total hepatic Mu-class GST (from specimen L4), purified by affinity-chromatograph hydroxyapatite chromatography, were resolved by chromatofocusing as descrit section 4.04d. The column (1.6 cm x 32 cm), containing polybuffer exchanger PE was pre-equilibrated in 25 mM Tris/$CH_3COOH$ buffer pH 8.9 and following the appli of protein sample, a pH gradient was formed using polybuffer 96 at pH 5.5. The p determined at 4 °C (▪) in every fifth fraction collected. GST activity, with 1-chlor dinitrobenzene as substrate (▲), and protein concentrations were measured (○). fractions which were combined are indicated by the horizontal bars.

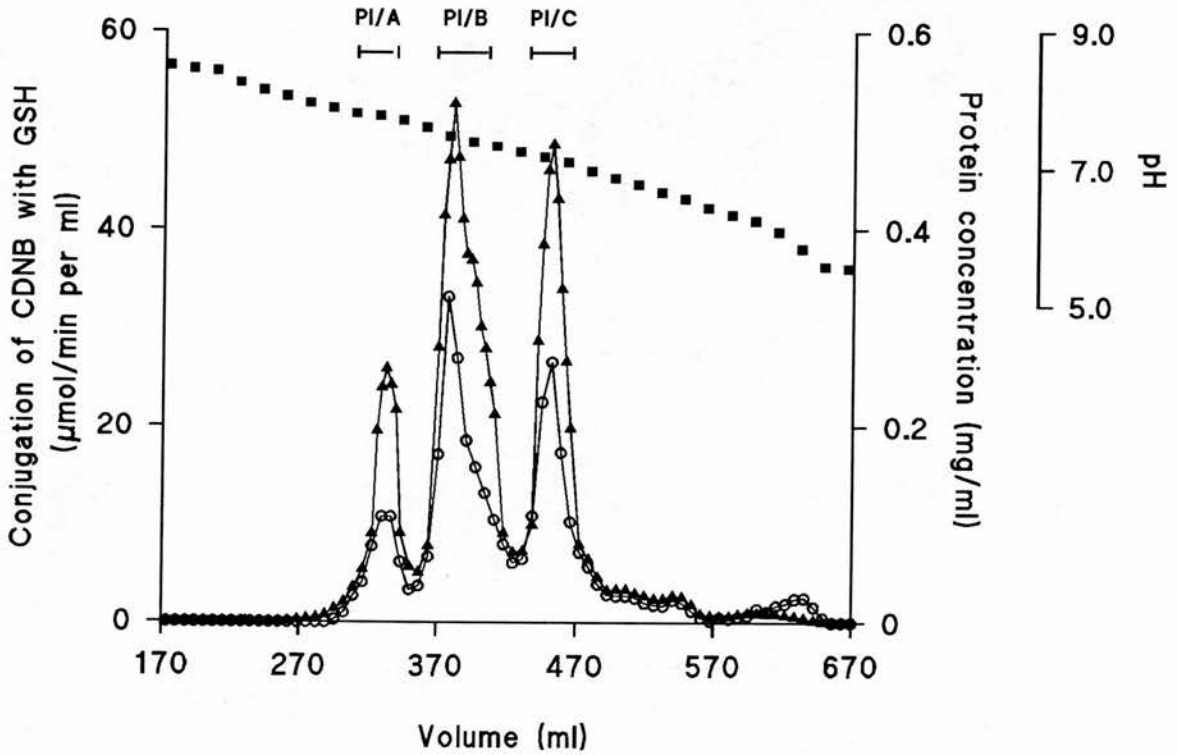

PI/A
PI/B
PI/C
60
40
20
0
0.6
0.4
0.2
0
9.0
7.0
5.0
pH
Conjugation of CDNB with GSH
(µmol/min per ml)
Protein concentration (mg/ml)
170
270
370
470
570
670
Volume (ml)

**Figure 7.02**

**Table 7.01 Purification of human hepatic Mu-class GST isoenzymes**

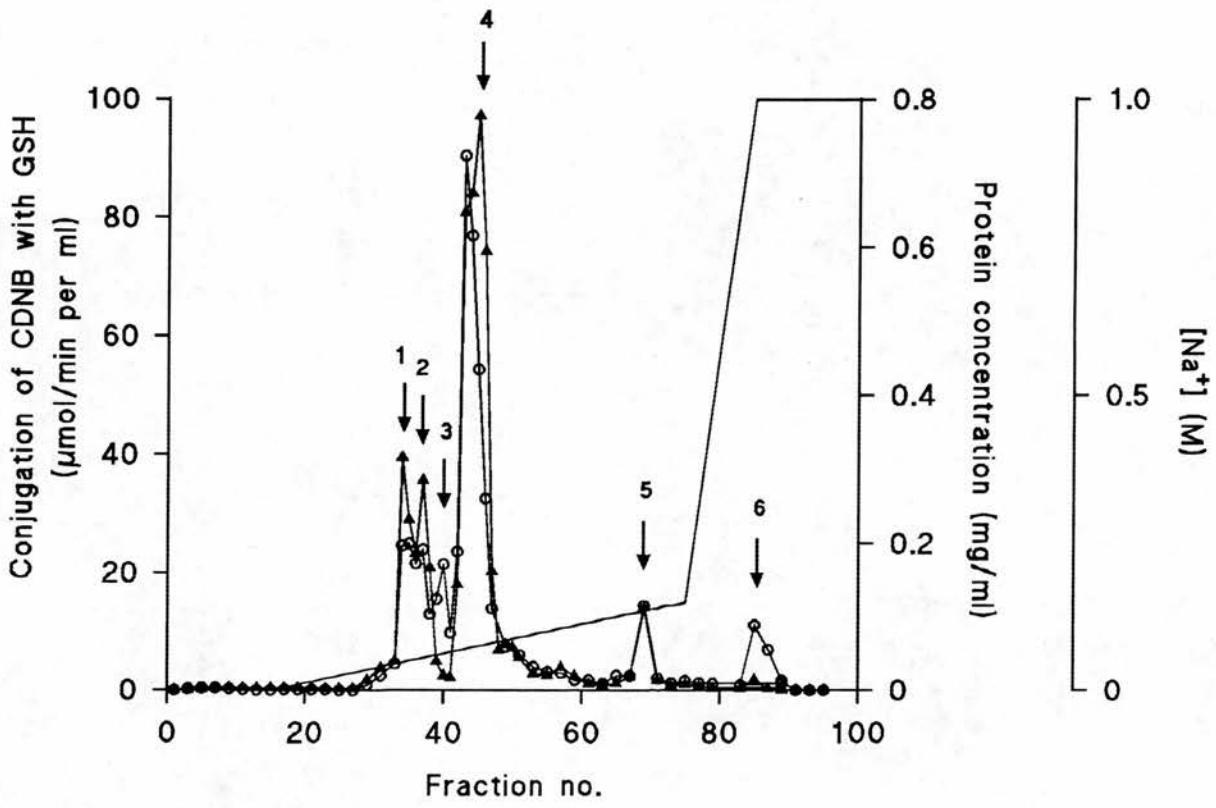
For experimental details see section 4.09. GST activity was measured with 1-chloro-2,4-dinitrobenzene at 37 °C.

| Fraction | Total protein (mg) | Total activity ($\mu$mol/min) | Specific activity ($\mu$mol/min per mg) | Recovery (%) |
|---|---|---|---|---|
| Cytosol | 25620 | 19156 ± 180.0 | 0.75 ± 0.01 | 100 |
| Affinity-chromatography pool | 273.6 | 13440 ± 65.0 | 49.1 ± 0.24 | 70.2 |
| Hydroxyapatite chromatography pools: | | | | |
| PI | 28.1 | 4119 ± 36.8 | 146.4 ± 1.3 | 21.5 |
| PII | 162.0 | 6356 | 39.2 | 33.2 |
| Chromatofocusing,pH range 8.5-5.5, pools: | | | | |
| PI/A ($N_1^aN_1^a$) | 1.5 | 285.2 ± 3.9 | 190.1 ± 2.6 | 1.49 |
| PI/B ($N_1^aN_1^b$) | 4.1 | 667.4 ± 15.2 | 162.8 ± 3.7 | 3.48 |
| PI/C ($N_1^bN_1^b$) | 3.6 | 619.3 ± 9.8 | 172.0 ± 2.7 | 3.23 |

**Figure 7.03 Analysis by Mono Q f.p.l.c. and SDS/PAGE of human GST isoen pools purified from skeletal muscle and cerebrum samples fro same individual**

Anion-exchange chromatography on Mono Q f.p.l.c. of S-hexylglutathione-affinity-pu GST from a skeletal muscle sample (M4) and a cerebrum sample (C4), obtained fro same individual (see Table 4.01) was performed as described in section 4.06a. P a and c show the elution profiles resulting from chromatography of material purifiec samples M4 and C4 respectively. The Mono Q columns were each developed wit 1.0 M NaCl gradient, established in two stages, as shown by the straight lines. activity, with 1-chloro-2,4-dinitrobenzene as substrate (▲), and protein concentrations measured (○). The protein-containing fractions which are indicated by arrows analysed by SDS/PAGE (panels b and d). The first lane on each gel contains the t hexylglutathione-affinity-purified material from each tissue specimen (TOT; 6 $\mu$g), a following lanes contain the fractions resulting from Mono Q f.p.l.c. as shown. The h GST markers $\mu$ ($M_r$ 26700) and $\pi$ ($M_r$ 24800) were run in parallel and the mobili these subunits are indicated.

(a)




(b)

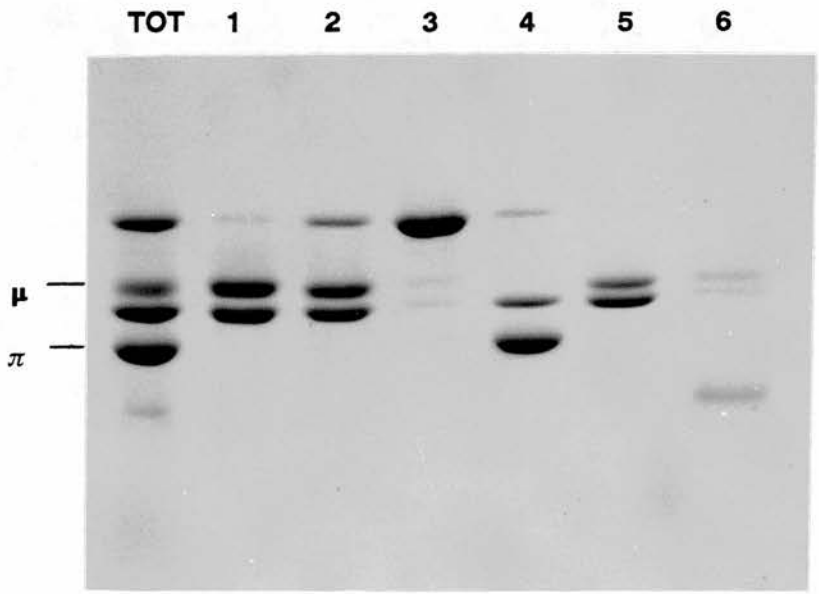
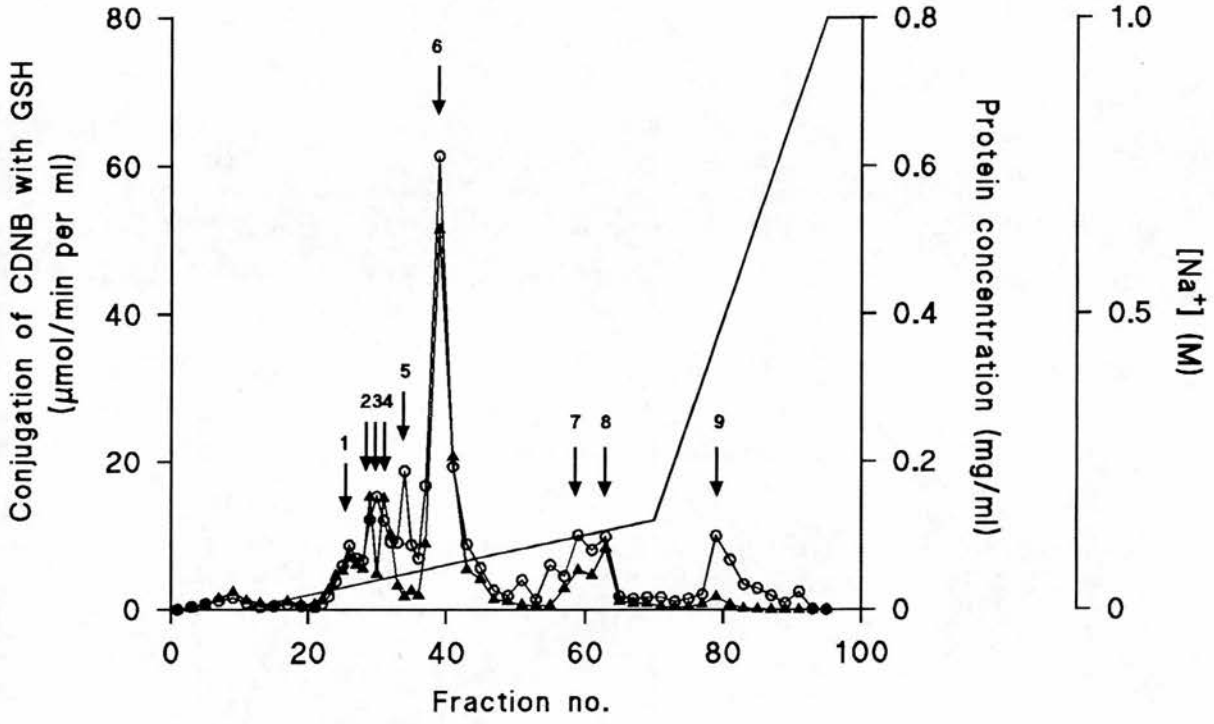



**Figure 7.03**

(c)




(d)

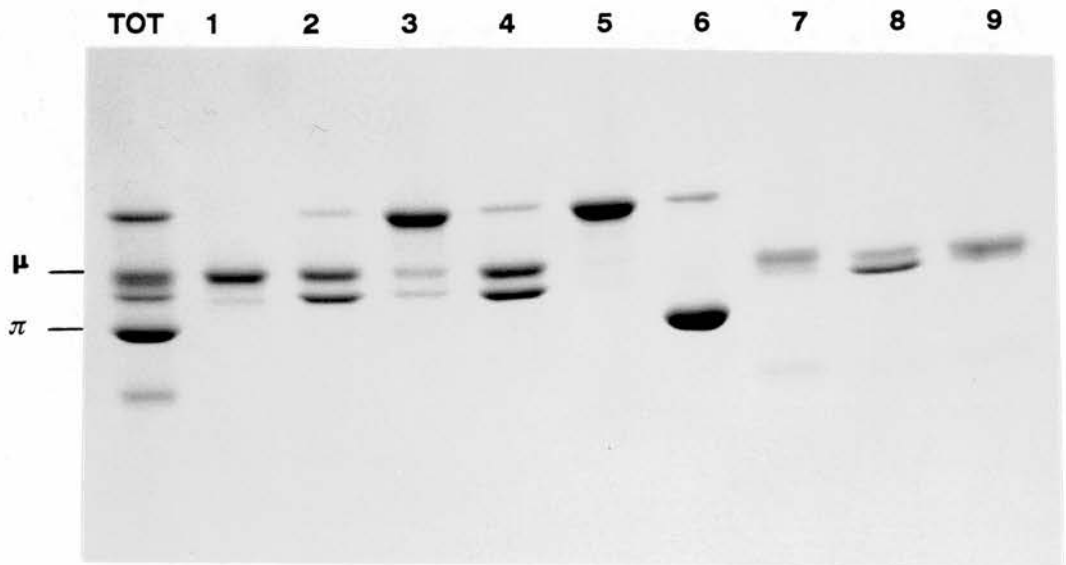



**Figure 7.03**

contained bands of subunit $M_r$ 26700 and 26000, together with a 28500-$M_r$ polypeptide; peak 3, which had no GST activity towards CDNB, contained the high molecular weight 28500-$M_r$ subunit; peak 4 contained three bands of $M_r$ 28500, 26000 and 24800; peak 5 contained two subunits of $M_r$ 26300 and 26000; and peak 6 contained the polypeptide bands of $M_r$ 26300 and 22500. The GST forms present in the peaks analysed can be identified from their electrophoretic and chromatographic properties. Hence, peaks 1 and 2 both contain $N_1$-type ($M_r$ 26700) and $N_2$ ($M_r$ 26000) subunits; peak 4 contains $N_2$ and Pi-class GST subunits ($M_r$ 24800); and peak 5 contains $N_2$ and $N_3$ ($M_r$ 26300) subunits.

When a cerebrum sample from the same individual was subjected to the same analytical procedure a more complex pattern of isoenzyme expression was observed (Figure 7.03c). Nine protein-containing fractions from the Mono Q column, seven of which had GST activity towards CDNB, were analysed by SDS/PAGE (Figure 7.03d). Those fractions which did not posses GST activity (3 and 5 as indicated) contained the 28500-$M_r$ polypeptide. Protein peak 1 contained $N_1$-type subunits ($M_r$ 26700); peaks 2 and 4 both contained $N_1$-type and $N_2$ ($M_r$ 26000) subunits; peak 6, which contained the major GST form, yielded a subunit which co-migrated with the GST $\pi$ ($M_r$ 24800) standard; peak 7 contained a subunit of $M_r$ 25500: peak 8 contained $N_2$ and $N_3$ ($M_r$ 26300) subunits; and peak 9 contained $N_3$ subunits.

The chromatographic profiles obtained following anion-exchange f.p.l.c. of skeletal muscle and cerebrum samples from this individual, therefore, both posses two protein peaks (1 and 2 in muscle; 2 and 4 in cerebrum) which contain $N_1$-type and $N_2$ subunits. This indicates that two heterodimeric proteins formed by the combination of the $N_2$ subunit with either $N_1^a$ or $N_1^b$ subunits are expressed in these extra-hepatic tissues. The heterodimeric enzyme formed by the combination of $N_1$-type and $N_3$ subunits was difficult to identify; however, it may be represented by peak 7, obtained following chromatography of the cerebrum sample, which contained a $M_r$ 26500 polypeptide.

# Chapter 8 Characterization of human Mu-class GST

Until recently only four homodimeric Mu-class GST had been identified and characterized. These included the hepatic enzymes $\mu$, $\psi$ (Warholm *et al.*,1983; Hayes, 1989) and $\phi$ (Stockman and Hayes, 1987), and a skeletal muscle form called GST4 (Board *et al.*, 1988). The work presented in this thesis includes the purification of five extra-hepatic ($N_1^aN_2$, $N_1^bN_2$, $N_2N_2$, $N_2N_3$ and $N_3N_3$) and three hepatic ($N_1^aN_1^a$, $N_1^aN_1^b$ and $N_1^bN_1^b$) Mu-class GST isoenzymes which are formed by the association of four N-type subunits. In order to establish the molecular relationships between these forms the catalytic, immunochemical and structural properties of the multiple human Mu-class GST have been investigated.

## 8.01 Physical properties of hepatic and extra-hepatic Mu-class GST

Analytical isoelectric focusing in thin-layer polyacrylamide slab gels was carried out on all of the purified Mu-class GST (Figure 8.01). All of the isoenzymes focused into single bands, thereby confirming their purity and allowing a pI value to be assigned to each GST.

SDS/PAGE analysis of the purified Mu-class isoenzymes is shown in Figure 8.02. In order to permit the designation of molecular mass values to these enzymes the human subunit markers $B_1B_2$ ($M_r$ 26000), $\mu$ ($M_r$ 26700) and $\pi$ ($M_r$ 24800) were used to calibrate the gel. The homodimeric enzymes $N_1^aN_1^a$, $N_1^bN_1^b$, $N_2N_2$ and $N_3N_3$ all gave a single band following electrophoresis. A close examination of the polyacrylamide gel revealed that $N_1^bN_1^b$ migrated slightly faster than $N_1^aN_1^a$ and their apparent $M_r$ values were calculated as 26600 and 26700 respectively. The protein bands present in the heterodimeric GST $N_1^aN_1^b$ were not resolved by SDS/PAGE, unlike the subunits which comprised the heterodimers $N_1^bN_2$ and $N_2N_3$ which were clearly separated by this technique. Table 8.01 shows the pI and $M_r$ values assigned to the purified Mu-class GST.

**Figure 8.01 Isoelectric focusing of human hepatic and extra-hepatic Mu-class isoenzymes**

Isoelectric focusing (IEF) was performed in a broad range gels, pH 3.5-9.5, in 5% polyacrylamide. Panels a, b and c show the electrophoretic analysis of eight huma class GST (7-10 $\mu$g protein loaded). Protein calibration standards (STD) were run on gel and the pI values of these marker proteins are indicated. Isoenzymes $N_1{}^aN_1{}^a$, $N_1{}^aN_1{}^b$ and $N_1{}^bN_1{}^b$ (panel a) were purified from liver sample L4 by S-hexylglutathione-a chromatography followed by hydroxyapatite chromatography and chromatofocusi the pH range 8.5-5.5, as described in section 4.09. $N_1{}^bN_2$ and $N_2N_2$ (panel a) isolated from skeletal muscle sample M3 by a combination of affinity chromatograph anion-exchange chromatography on Mono Q f.p.l.c followed by either chromatofoc in the case of $N_1{}^bN_2$, or hydroxyapatite h.p.l.c. in the case of $N_2N_2$. Details o purification of the skeletal muscle enzymes are described in section 4.07. The purific of the testicular GST $N_3N_3$ (panel a), described in section 4.08, involved a chromatography followed by Mono Q f.p.l.c. Panel b shows isoelectric focusing of which was purified from skeletal muscle sample M2 by affinity chromatography, a exchange chromatography on Mono Q followed by chromatofocusing on Mo Isoenzyme $N_2N_3$ (panel c) was purified from skeletal muscle sample M1 by a combi of affinity chromatography and anion-exchange chromatography on Mono Q.

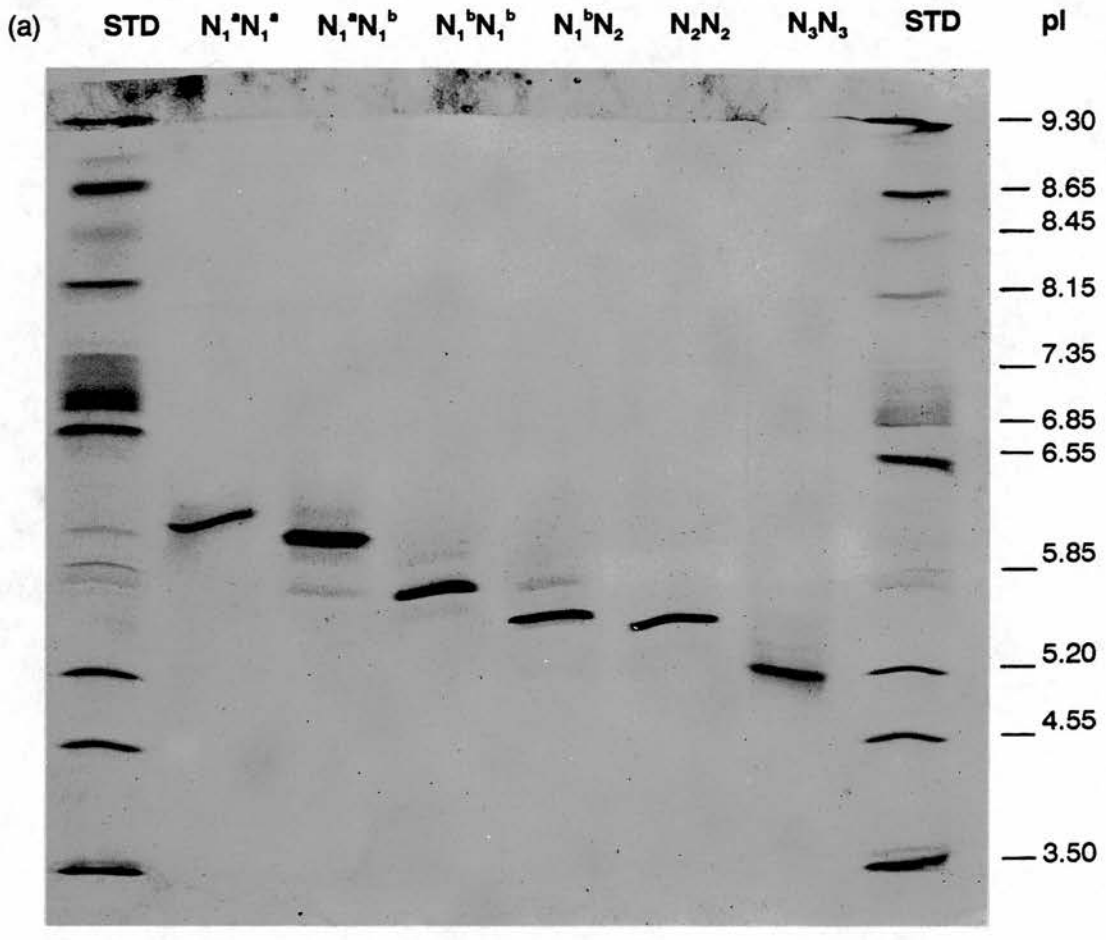

(a)
STD
$N_1^aN_1^a$
$N_1^aN_1^b$
$N_1^bN_1^b$
$N_1^bN_2$
$N_2N_2$
$N_3N_3$
STD
pI
9.30
8.65
8.45
8.15
7.35
6.85
6.55
5.85
5.20
4.55
3.50

**Figure 8.01**

(b)

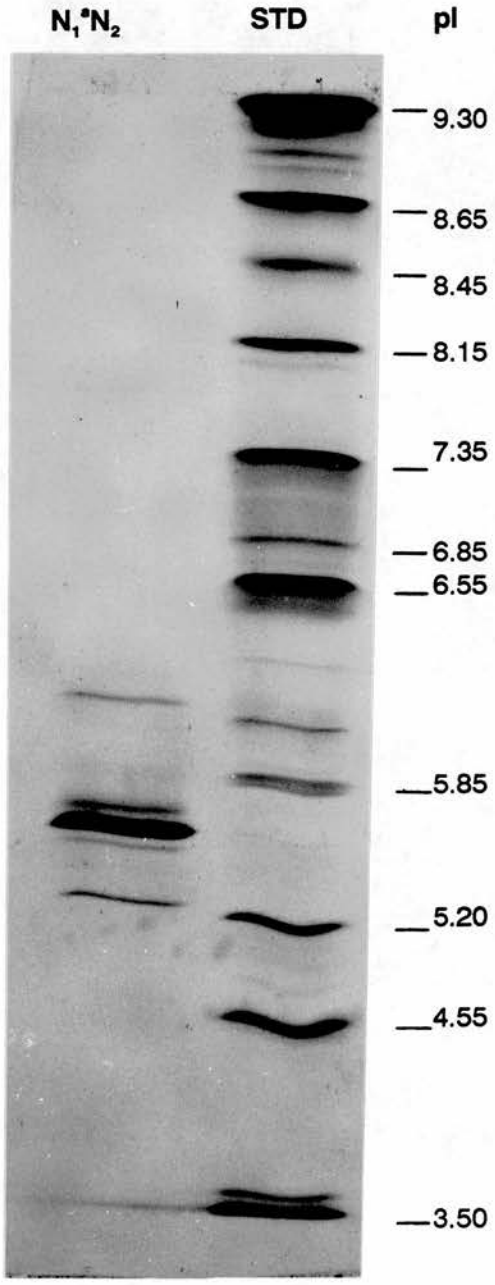



**Figure 8.01**

(c)

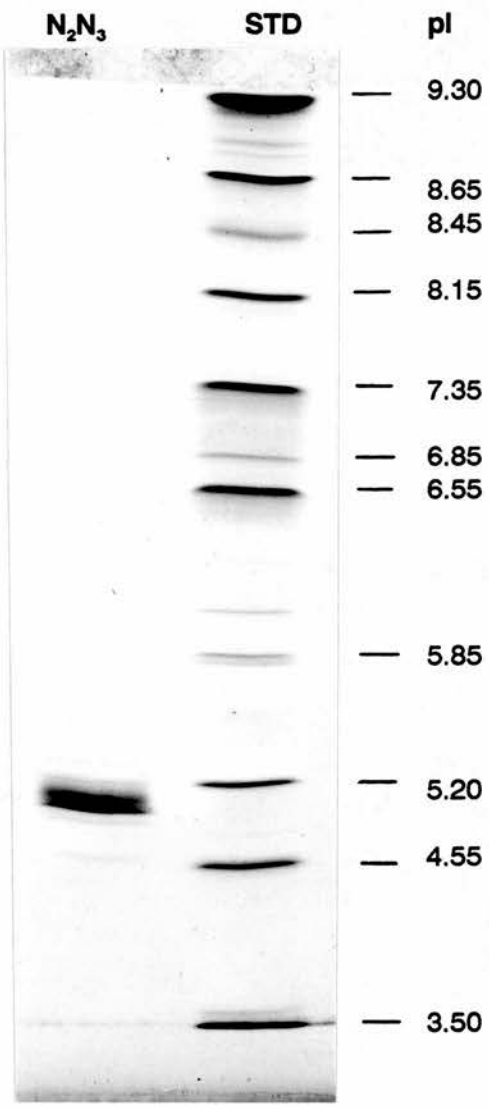



**Figure 8.01**

**Figure 8.02 SDS/polyacrylamide-gel electrophoresis of human Mu-class isoenzymes**

SDS/PAGE analysis was performed, according to the method of Laemmli (1970), us resolving gel which contained 12% (w/v) polyacrylamide and 0.32% (w/v) N,N'-methy bisacrylamide. The human GST subunit markers $B_1B_2$ ($M_r$ 26000), $\mu$ ($M_r$ 26700) and 24800) were loaded into the first three tracks. The following seven tracks cont human Mu-class GST purified from liver sample L4 ($N_1^aN_1^a$, $N_1^aN_1^b$ and $N_1^bN_1^b$), sk muscle samples M3 ($N_1^bN_2$ and $N_2N_2$) and M4 ($N_2N_3$), and testis sample T3 ($N_3$ indicated. The purification procedures used are described both briefly in the lege Figure 8.01 and in detail in sections 4.07, 4.08 and 4.09.

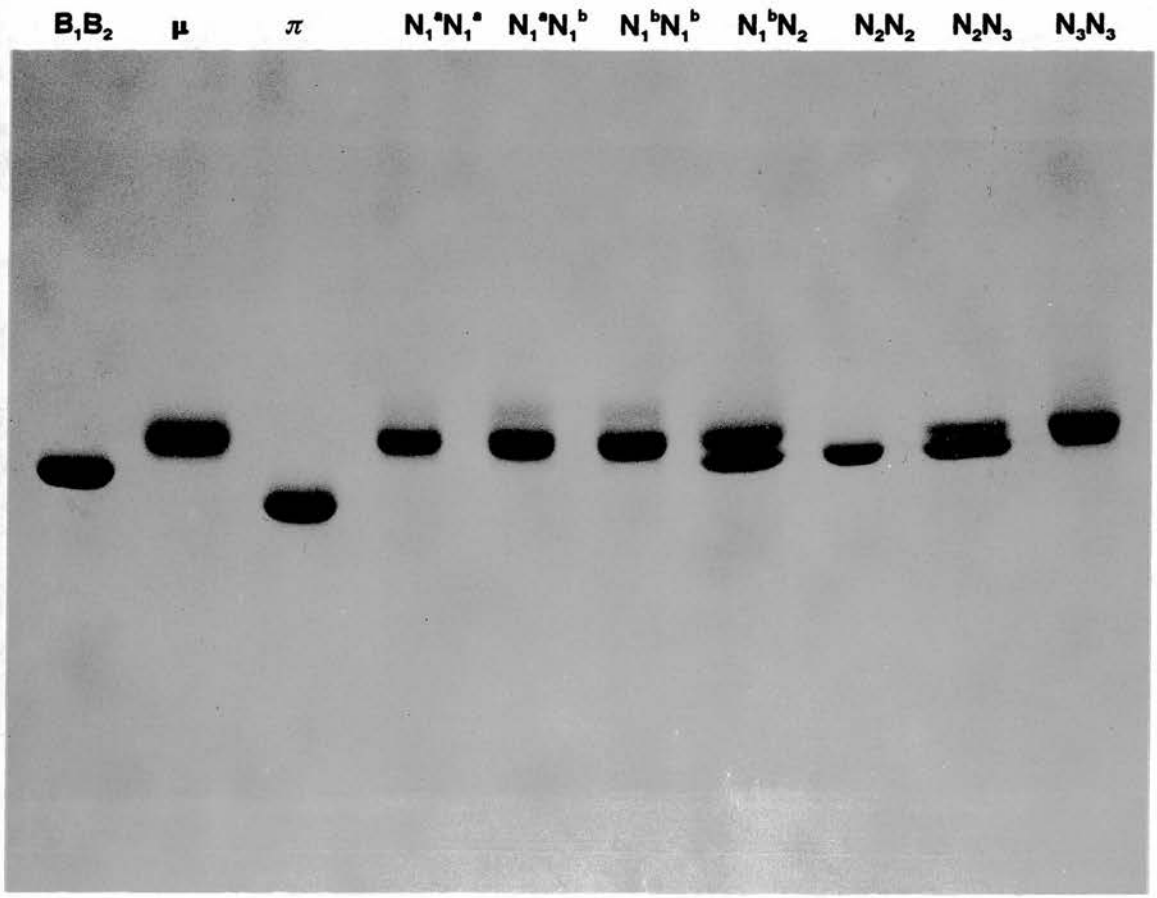



**Figure 8.02**

**Table 8.01 Physical properties of human Mu-class GST isoenzymes**

| Enzyme | Subunit $M_r$ | pI value |
|---|---|---|
| $N_1^aN_1^a$ ($\mu$) | 26700 | 6.10 |
| $N_1^aN_1^b$ | 26700/26600 | 5.90 |
| $N_1^bN_1^b$ ($\psi$) | 26600 | 5.55 |
| $N_1^aN_2$ | 26700/26000 | 5.65 |
| $N_1^bN_2$ | 26600/26000 | 5.40 |
| $N_2N_2$ | 26000 | 5.35 |
| $N_2N_3$ | 26000/26300 | 5.00 |
| $N_3N_3$ | 26300 | 5.00 |

## 8.02 Immunological properties of human Mu-class GST

Western blot analysis of skeletal muscle GST subunits is shown in Figure 8.03. The subunits which comprise the isoenzymes $N_2N_2$ and $N_2N_3$, and those from peak P3-A, isolated on hydroxyapatite h.p.l.c., were purified on reverse-phase h.p.l.c. before being probed with antisera raised against the human GST $B_1B_1$, $\mu$ and $\pi$. GST subunits $N_2$ and $N_3$ cross-reacted with anti-($\mu$) IgG but not with antisera raised against either Alpha- or Pi-class GST. By contrast, the component of peak P3-A was found to cross-react with antibodies to Pi-class GST but not with other antibodies against other GST.

Purified human Mu-class GST were also challenged with antisera raised against both human and rat Mu-class isoenzymes, in an attempt to differentiate between the $N_1$-type, $N_2$ and $N_3$ subunits (Figure 8.04). No immunochemical differences were apparent between GST $N_1^aN_1^a$ and $N_1^bN_1^b$. These hepatic enzymes cross-reacted strongly with antibodies raised against human GST $N_1^aN_1^a$ (or $\mu$) and $N_2N_2$, and rat GST $Yb_1Yb_1$, YnYn and YoYo, however, no cross-reactivity was observed between these enzymes and anti-($N_3N_3$) IgG. The isoenzyme purified from skeletal muscle, GST $N_2N_2$, was immunochemically related to the isoenzymes containing $N_1$-type subunits. However, when the Mu-class GST were probed with anti-($N_1^aN_1^a$) IgG, differences in the intensity of the peroxidase staining was observed between the $N_1$-type and the $N_2$ subunits. The $N_1$-type subunits also cross-reacted to a greater extent, than the $N_2$ subunits, with antisera raised against the various rat Mu-class GST.

**Figure 8.03 Cross-reactivity of skeletal muscle GST subunits with antisera against human Alpha, Mu and Pi-class GST**

Immunochemical analysis of individual skeletal muscle GST subunits was performed the Western Blot technique described by Towbin *et al.* (1979). GST $N_2N_2$, $N_2N_3$ and p purified from skeletal muscle sample M1, as described in section 4.07, and the su present in these isoenzymes were separated by reverse-phase h.p.l.c. using Bondapak column developed with a 40-55% (v/v) acetonitrile gradient in 0.1% trifluoroacetic acid. SDS/polyacrylamide gels were loaded as follows; tracks 1, 2 human GST standards $B_1B_2$, $\mu$ and $\pi$; tracks 4 and 5, skeletal muscle Pi-class sub track 6 the $N_2$ subunit obtained following reverse-phase h.p.l.c. of $N_2N_2$; track 7 subunit isolated from $N_2N_3$ by the same technique; tracks 8 and 9, the $N_3$ subunit res following reverse-phase h.p.l.c. of $N_2N_3$. The proteins were transferred electrophore onto nitrocellulose paper and probed for cross-reactivity with specific antisera against human GST $B_1B_1$ (panel a), GST $\mu$, or $N_1^aN_1^a$, (panel b) and GST $\pi$ (pane

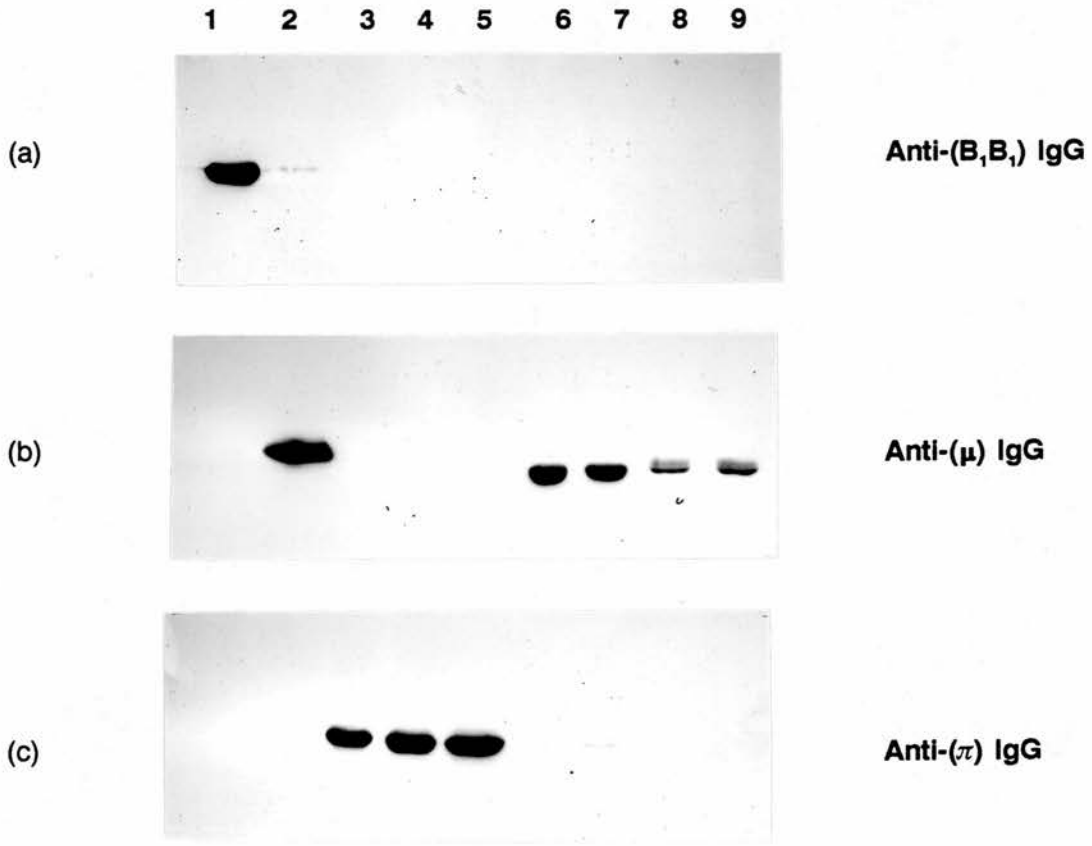



**Figure 8.03**

**Figure 8.04 Immunoblot analysis of human Mu-class GST purified from sk muscle, testis and liver**

Purified hepatic and extra-hepatic human Mu-class GST were subjected to immu analysis (Towbin *et al.*, 1979) using antisera raised against human Mu-class GST (a), $N_2N_2$ (b) and $N_3N_3$ (c) and rat Mu-class GST $Yb_1Yb_1$ (d), YnYn (e) and YoYo (f). F (1 $\mu$g/band) was applied to SDS/polyacrylamide gels as follows; track 1, the a against which the relevant antiserum was raised; tracks 2, 3 and 4, human GST B and $\pi$ standards; tracks 5 and 6, the hepatic (L4) GST $N_1{}^aN_1{}^a$ and $N_1{}^bN_1{}^b$; track 7, s muscle (M3) GST $N_1{}^bN_2$; track 8, skeletal muscle (M3) GST $N_2N_2$; track 9, skeletal m (M4) GST $N_2N_3$; track 10, testicular (T3) GST $N_3N_3$.

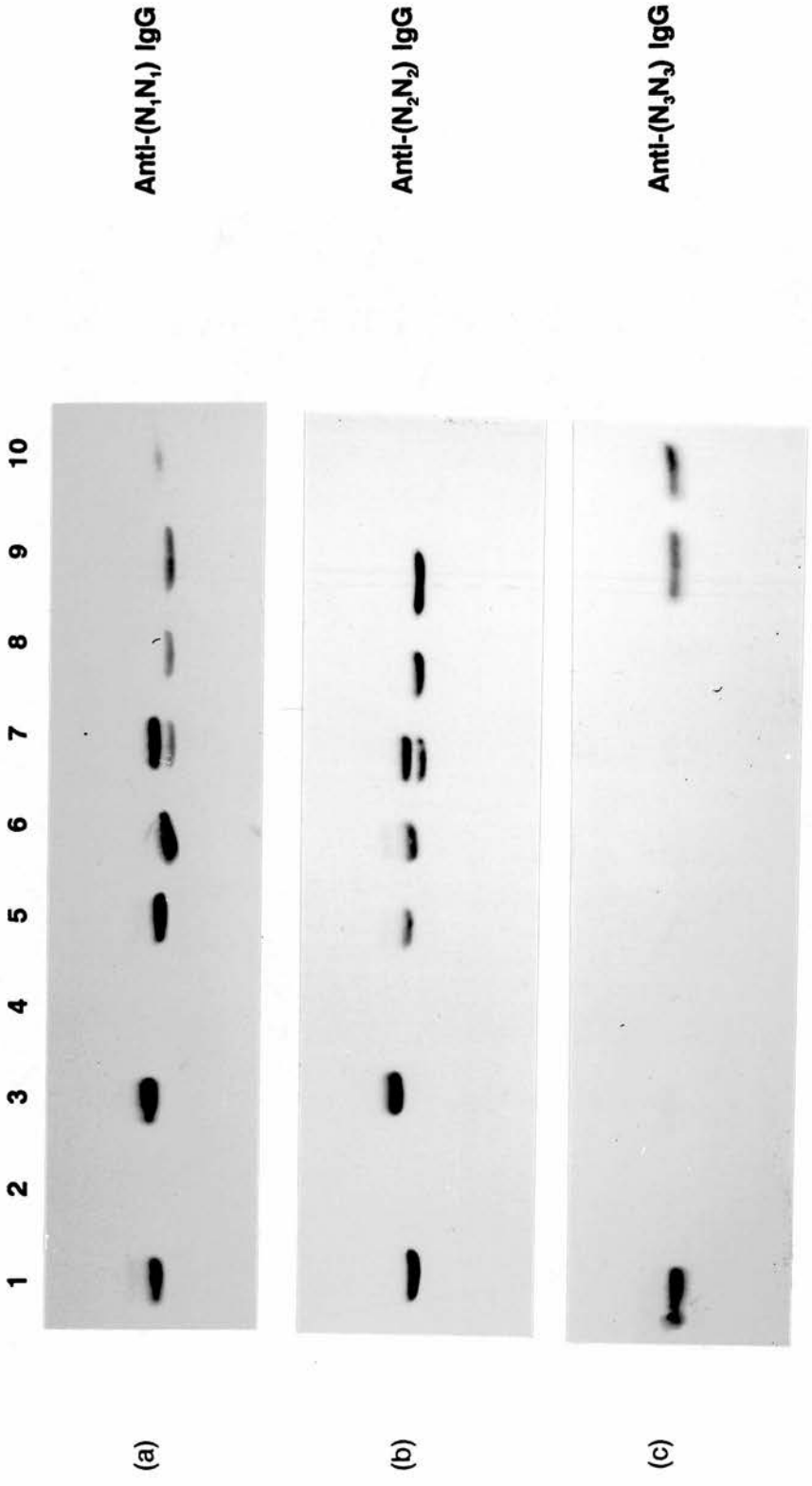



**Figure 8.04**

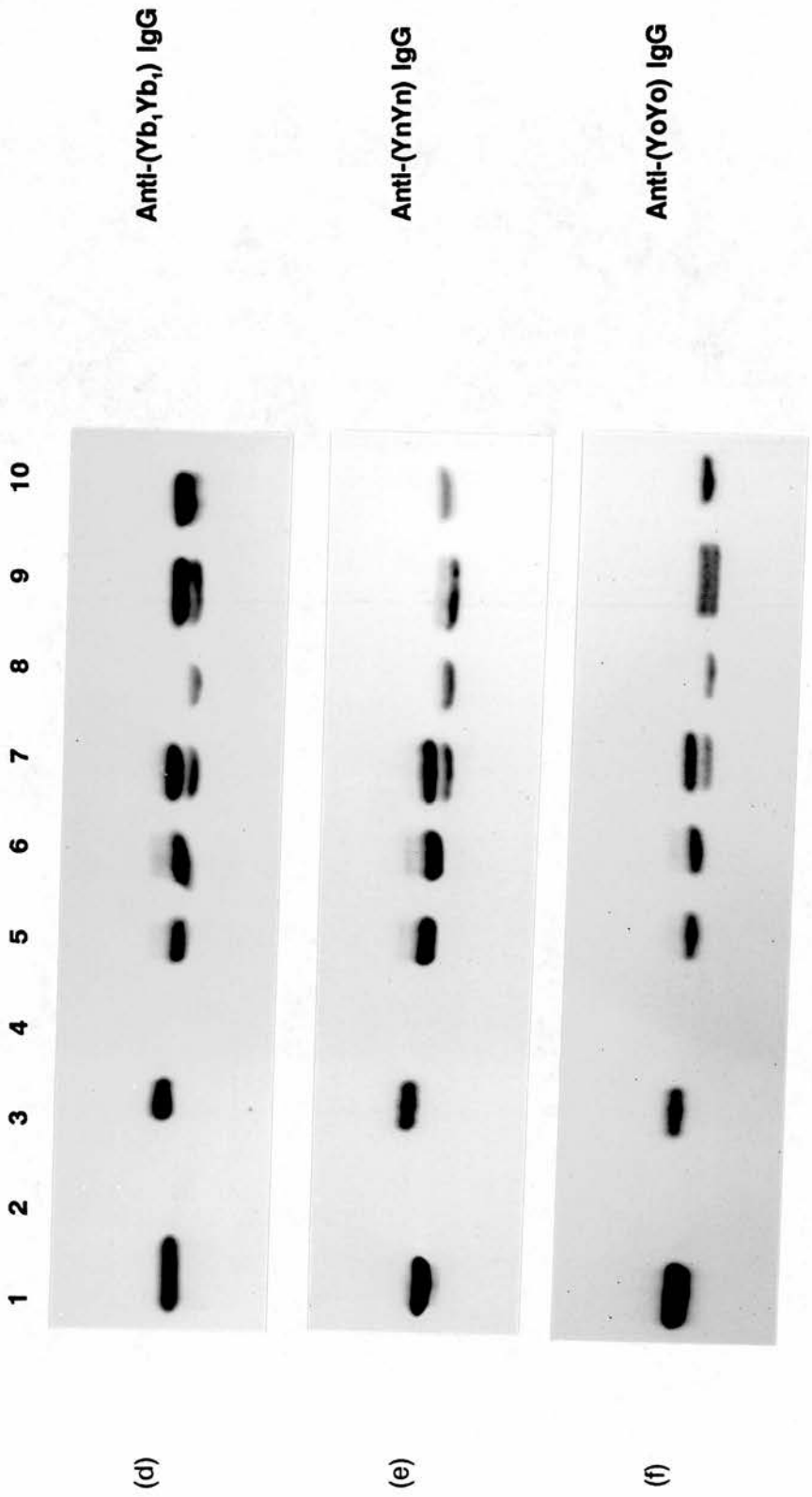



**Figure 8.04**

The subunit which comprises the testis enzyme GST $N_3N_3$ was immunochemically distinct from the other human Mu-class subunits. No cross-reactivity was observed between GST $N_3N_3$ and antisera raised against GST $N_2N_2$, and only a very slight cross-reactivity was apparent when this GST was probed with anti-($N_1^aN_1^a$) IgG. When the human Mu-class GST were challenged with antisera raised against GST $N_3N_3$ the only apparent cross-reactivity occurred with the immunogen and the $N_3$ subunit component of GST $N_2N_3$. An immunochemical relationship, however, existed between GST $N_3N_3$ and the rat Mu-class GST $Yb_1Yb_1$, YnYn and YoYo; the strongest cross-reactivity being apparent between this enzyme and with antisera raised against GST $Yb_1Yb_1$ and YoYo.

## 8.03 Catalytic properties of human Mu-class enzymes

The specific activities of the human Mu-class GST, purified from liver skeletal muscle and testis, and a Pi-class enzyme, purified from skeletal muscle, with a range of substrates are shown in Table 8.02. All of the enzymes had activity towards the substrate 1-chloro-2,4-dinitrobenzene, however, the activity of the testis enzyme was considerably lower than that of the other GST. Different patterns of activity were also observed when *trans*-4-phenyl-3-buten-2-one, 1,2-dichloro-4-nitrobenzene and *para*-nitrobenzyl chloride were employed as substrates for these enzymes. The homodimeric GST $N_2N_2$ and the heterodimeric GST $N_2N_3$ both had activity with 1,2-dichloro-4-nitrobenzene but no detectable activity towards either *trans*-4-phenyl-3-buten-2-one or *para*-nitrobenzyl chloride, unlike the hepatic enzymes, $N_1^aN_1^a$, $N_1^aN_1^b$ and $N_1^bN_1^b$, where the converse was true. The heterodimeric GST $N_1^bN_2$, however, had activity towards all three of these substrates. None of the enzymes examined had activity with bromosulphophthalein as substrate. The hydroxyalkenals, 4-hydroxynonenal and 4-hydroxydecinal, however, were good substrates for all of the Mu-class GST. Those isoenzymes which contained $N_1$-type subunits had the highest activity towards cumene hydroperoxide and the skeletal muscle Pi-class enzyme (P3-A) was the most active towards ethacrynic acid.

## 8.04 Structural properties of human Mu-class GST

### 8.04a N-Terminal amino acid sequence analysis

The GST subunits which comprise the skeletal muscle isoenzymes $N_1^aN_2$, $N_2N_2$ and $N_2N_3$ were subjected to N-terminal sequence analysis in order to help establish the molecular relationship between the muscle enzymes and to provide evidence that the polypeptide

**Table 8.02 Specific activities of Mu-class isoenzymes**

For experimental details see section 3.02c. All enzyme assays were performed at 37 °C. Results are expressed as means ± S.D. for three determinations. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; *t*-PBO,*trans*-4-phenyl-3-buten-2-one; DCNB,1,2-dichloro-4-nitrobenzene; BSP, bromosulphophthalein; *p*-NBC, *para*-nitrobenzyl chloride; 4-HNE, 4-hydroxynonenal; 4-HDE, 4-hydroxydecinal; CuOOH, cumene hydroperoxide; EA, ethacrynic acid; SM Pi, skeletal muscle Pi-class enzyme; N.D., not detected.

| Enzyme | Specific activity ($\mu$mol/min per mg of protein) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CDNB | *t*-PBO | DCNB | BSP | *p*-NBC | 4-HNE | 4-HDE | CuOOH | EA |
| $N_1^aN_1^a$ | 190.2 ± 2.6 | 0.205 ± 0.039 | N.D. | N.D. | 2.66 ± 0.19 | 3.33 ± 0.22 | 3.29 ± 0.13 | 0.296 ± 0.001 | 0.47 ± 0.04 |
| $N_1^aN_1^b$ | 161.4 ± 3.7 | 0.130 ± 0.01 | N.D. | N.D. | 2.20 ± 0.14 | 2.26 ± 0.07 | 1.93 ± 0.08 | 0.275 ± 0.018 | 0.05 ± 0.02 |
| $N_1^bN_1^b$ | 172.0 ± 2.7 | 0.164 ± 0.016 | N.D. | N.D. | 2.18 ± 0.25 | 2.50 ± 0.06 | 2.30 ± 0.12 | 0.282 ± 0.018 | 0.12 ± 0.05 |
| $N_1^bN_2$ | 203.1 ± 4.4 | 0.128 ± 0.004 | 1.71 ± 0.03 | N.D. | 2.64 ± 0.12 | 2.97 ± 0.26 | 2.82 ± 0.25 | 0.258 ± 0.005 | 0.24 ± 0.06 |
| $N_2N_2$ | 276.8 ± 10.4 | N.D. | 1.99 ± 0.24 | N.D. | N.D. | 3.62 ± 0.38 | 2.99 ± 0.17 | 0.037 ± 0.001 | 0.29 ± 0.06 |
| $N_2N_3$ | 171.9 ± 1.8 | N.D. | 2.09 ± 0.11 | N.D. | N.D. | 3.33 ± 0.28 | 3.17 ± 0.23 | 0.134 ± 0.026 | 0.48 ± 0.04 |
| $N_3N_3$ | 15.2 ± 0.1 | N.D. | N.D. | N.D. | N.D. | 1.82 ± 0.11 | 1.87 ± 0.19 | 0.053 ± 0.016 | 0.31 ± 0.08 |
| SM Pi | 103.4 ± 1.4 | N.D. | N.D. | N.D. | N.D. | 1.58 ± 0.35 | 0.40 ± 0.02 | N.D. | 1.24 ± 0.08 |

of $M_r$ 26000, that has been designated $N_2$, is indeed a common subunit. Before sequence analysis the $N_2$ and $N_3$ subunits, from GST $N_2N_2$ and $N_2N_3$, were isolated using reverse-phase h.p.l.c., as described in section 5.05. The results of these analyses are shown in Figure 8.05.

The subunits of $M_r$ 26000 present in the $N_2N_2$ homodimer and the $N_2N_3$ heterodimer were shown to have identical N-terminal sequences over the first ten residues. Although the subunits in GST $N_1{}^aN_2$ could not be separated on the μ Bondapak $C_{18}$ column it was possible to interpret the sequence data obtained from the heterodimeric protein over most the residues analysed. The direct sequencing of GST $N_1{}^aN_2$ yielded unambiguous assignments from cycles 1, 2, 4, 5, 6, 7, 9, 10 , 11, 12, 13 and 14, but at residues 3, 8 and 15 the Sequenator identified two amino acids, namely threonine or isoleucine at cycle 3, asparagine or aspartic acid at cycle 8 and serine or alanine at cycle 15. When these sequences were compared with those of human Mu-class GST described in the literature, it was apparent that one sequence could be attributed to that of human liver GST μ or ψ (Alin *et al.*, 1985a; Hayes, 1989) and the other was identical with the 24 residues published for the skeletal muscle enzyme GST4 (Board *et al.*, 1988). These data suggest that the subunit of $M_r$ 26000, designated $N_2$, in GST $N_1{}^aN_2$, $N_2N_2$ and $N_2N_3$ represents the same polypeptide. The $N_3$ subunit that was purified by reverse-phase h.p.l.c. was found to posses a blocked N-terminus and could not be sequenced directly.

### 8.04b Primary structure of the Mu-class GST $N_2N_2$ and $N_3N_3$

Protein sequencing of the skeletal muscle enzyme GST $N_2N_2$ and the testicular GST $N_3N_3$ was possible following cleavage of these proteins with cyanogen bromide. When the resulting peptides, from the two enzymes, were resolved by reverse-phase h.p.l.c. on the μ Bondapak $C_{18}$ column markedly different profiles were obtained (Figures 8.06 and 8.07).

The protein sequence information obtained from the muscle Mu-class GST $N_2N_2$ has enabled the comparison of 52% of its primary structure with that of other human Mu-class enzymes reported in the literature. Four of the fragments CNBr-2, CNBr-5, CNBr-6 and CNBr-7, represented by peptide-containing peaks a, b, c and d respectively (Figure 8.06), were subjected to automated amino acid sequencing. In Figure 8.08 the amino acid sequences obtained from these peptides, together with the N-terminal sequence of subunit $N_2$ (CNBr-1), are aligned with the homologous sequences deduced from the

| Enzyme | Subunit(s) examined | Residue 1 5 10 15 20 |
|---|---|---|
| $N_1^aN_1^a(\mu)$ | $N_1^a$ | P M I L G Y W D I R G L A H A I R L L L E Y T D |
| $N_1^aN_2$ | $N_1^a + N_2$ | P M I L G Y W D I R G L A H A T N S |
| $N_2N_2$ | $N_2$ | P M T L G Y W N I R |
| $N_2N_3$ | $N_2$ | P M T L G Y W N I R G L A H S I R L L L E Y T D |
| $N_2N_3$ | $N_3$ | – |
| GST4 | | P M T L G Y W N I R G L A H S I R L L L E Y T D |

**Figure 8.05 N-terminal amino acid sequences of human N-type GST subunits**

Before sequence analysis the individual subunits from purified skeletal muscle isoenzymes $N_2N_2$ and $N_2N_3$ (sample M1) were isolated using reverse-phase h.p.l.c. as described in the legend to Figure 5.05. The subunits present in GST $N_1^aN_2$ (sample M2) could not be separated on the μ Bondapak column, therefore, this heterodimeric protein was analysed directly. N-terminal amino acid sequences were obtained following automated Edman degradation as described in section 3.04c. Subunit $N_3$ has a blocked N-terminus. The N-terminal amino acid sequences of GST μ (or $N_1^aN_1^a$) (Hayes, 1989) and GST4 (Board *et al.*, 1988) are aligned for comparison.

**Figure 8.06 Preparative peptide map of human GST $N_2N_2$**

Peptides were obtained following cyanogen bromide digest of GST $N_2N_2$ (1.0 mg), p
under reducing conditions, as described in section 3.04a. The CNBr-cleaved frag
were dissolved in 1.5 ml of aq. 0.1% (v/v) trifluoroacetic acid and, after centrifug
portions (0.5 mg of protein) were applied to a Waters μ Bondapak $C_{18}$ column (
particle size; 0.39 cm x 30 cm) that had been equilibrated with aq. 0.1%
trifluoroacetic acid. A linear gradient of 0-70% (v/v) acetonitrile in aq. 0.1%
trifluoroacetic acid, indicated by the straight line, was used to resolve the peptides
$A_{220}$ of the eluate was monitored continuously (___).

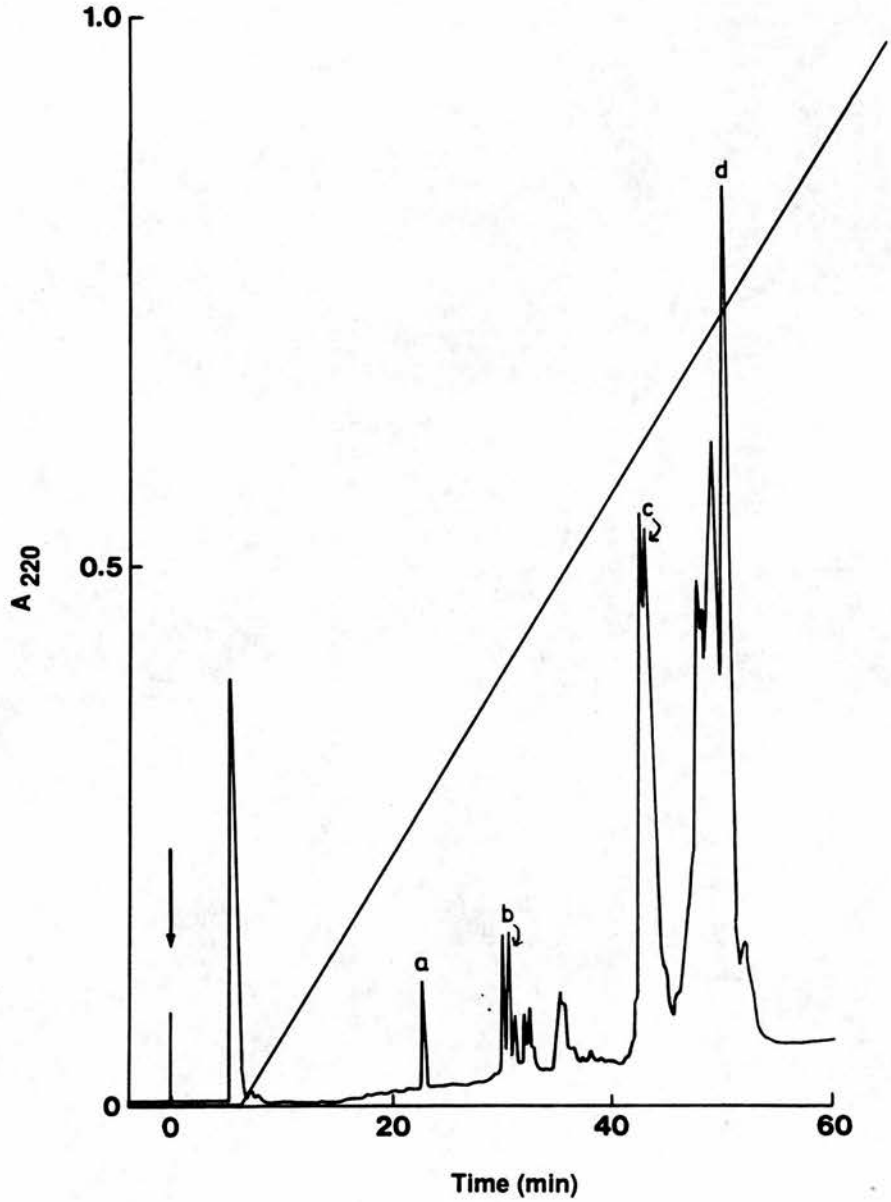



**Figure 8.06**

**Figure 8.07 Preparative peptide map of human GST $N_3N_3$**

A CNBr digest of a portion (0.7 mg) of purified testis GST $N_3N_3$ was prepared as des
in section 3.04a. The resulting CNBr-cleavage fragments were dissolved in 1.5 ml
0.1% (v/v) trifluoroacetic acid before application of 1.0ml to a reverse phase μ Bon
column, pre-equilibrated in aq. 0.1% (v/v) trifluoroacetic acid. The column was dev
with a 0-70% (v/v) acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid, as indica
the straight line. The $A_{220}$ of the column eluate was measured continuously (___)

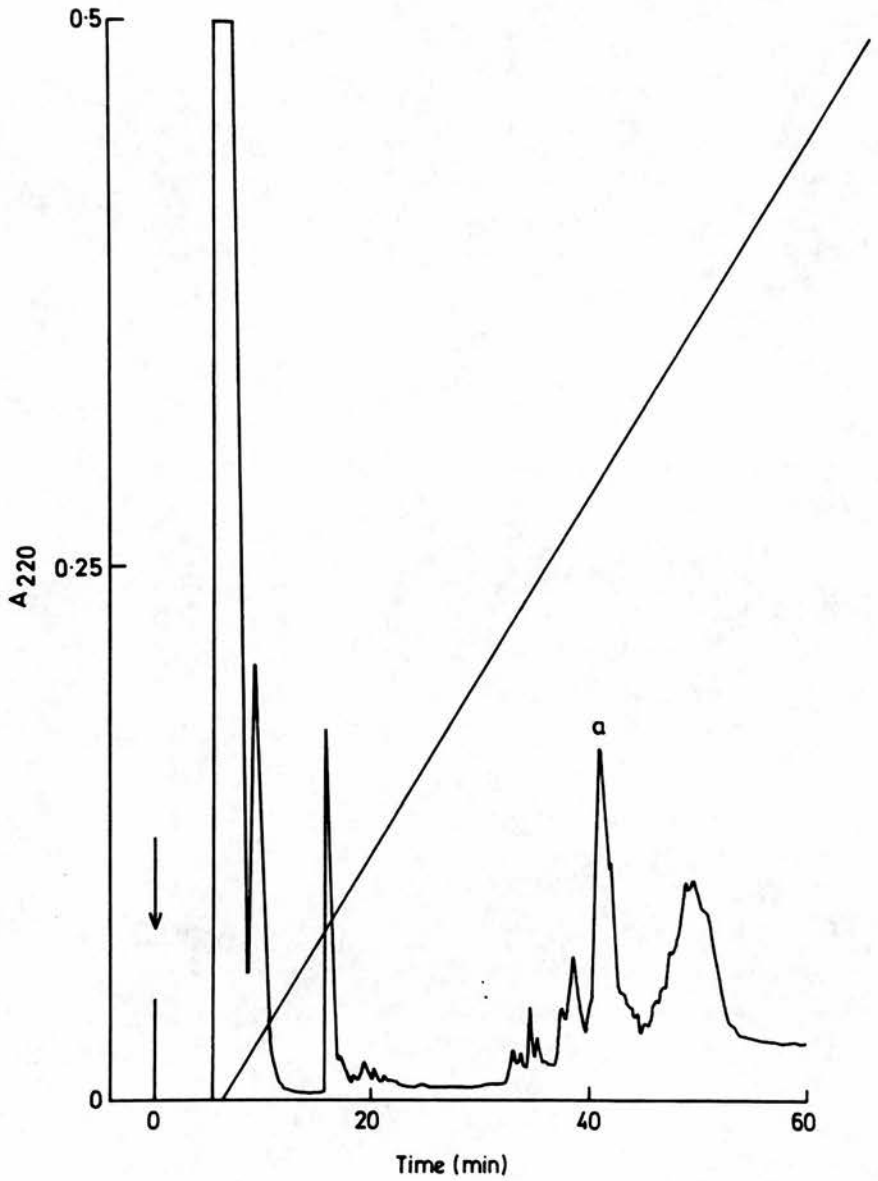



**Figure 8.07**

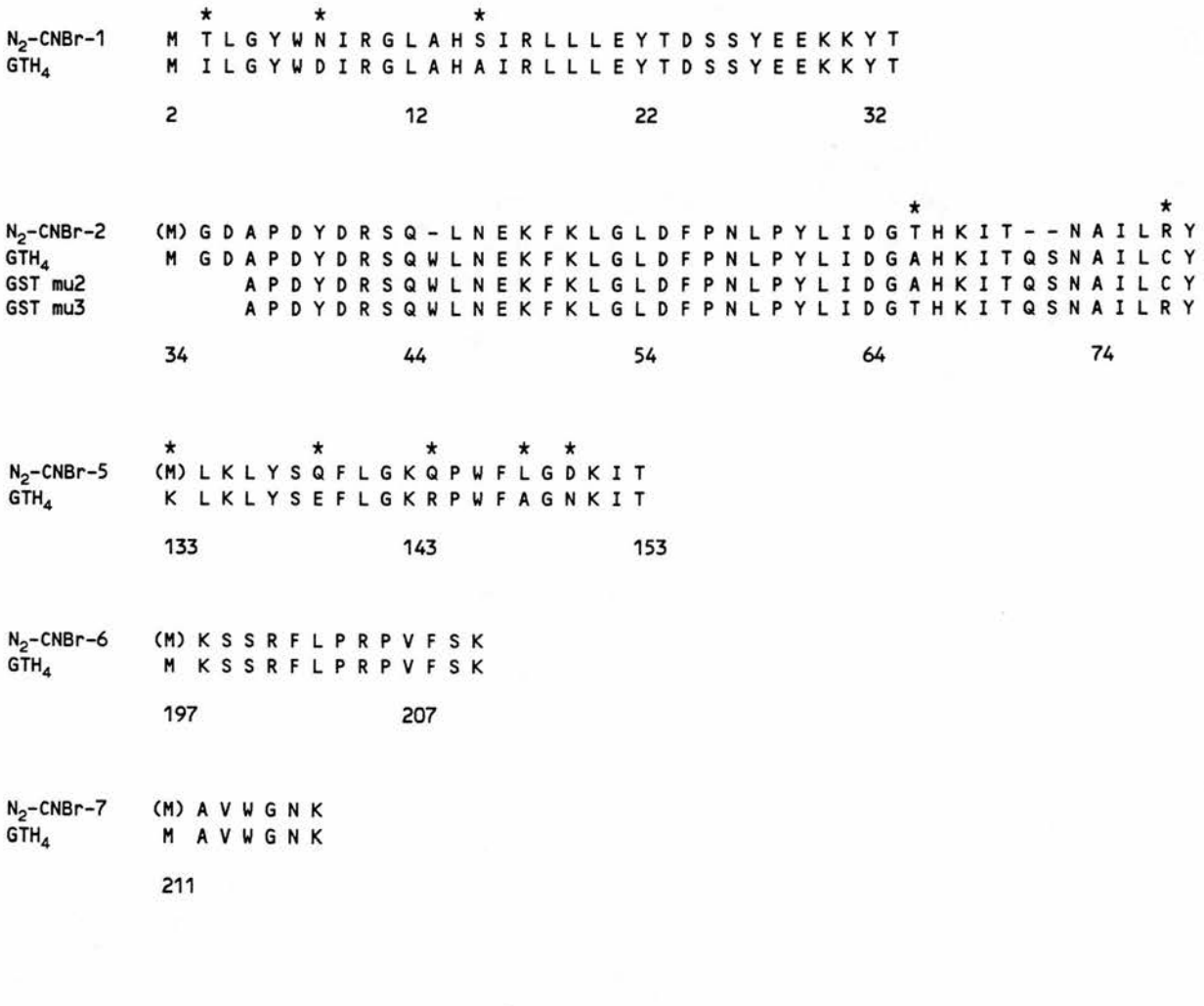



**Figure 8.08 Comparison of the primary structure of the $N_2$ subunit with those of other human Mu-class GST**

The N-terminal amino acid sequence (CNBr-1) and the sequences of the CNBr-derived peptides from subunit $N_2$ are aligned for comparison with the homologous sequences deduced from the cDNA $GTH_4$ (DeJong *et al.*, 1988) and the available homologous sequences deduced from the genomic DNA clones GST mu2 and GST mu3 (Taylor *et al.*, 1990, 1991). Putative methionine residues are in parentheses and a dash indicates where no residue could be identified with confidence. An asterisk (*) indicates amino acid residues that differ between the sequences.

human hepatic cDNA clone $GTH_4$, reported by DeJong *et al.* (1988), and the sequences deduced from the human Mu-class genomic clones, GST mu2 and GST mu3, described by Taylor *et al.* (1990, 1991).

Comparison of the N-terminal sequence of GST $N_2N_2$ (peptide CNBr-1) with that of the $GTH_4$ clone reveals the three amino acid differences found at positions 3, 8 and 15 predicted from the N-terminal sequence analysis of GST $\mu$ and GST $\psi$ discussed in section 8.04a. This is followed by a region of complete identity between the two sequences from residue 16 to residue 38. The peptide CNBr-2, which eluted from the $\mu$ Bondapak $C_{18}$ column at 44.5 min (peak c), was subjected to 45 automated Edman degradation cycles, allowing identification of residues 34-78. Over this region of the predicted protein amino acid sequence, information deduced from the genomic clones GST mu2 and GST mu3 is available for comparison with subunit $N_2$. Amino acid differences were apparent between the sequence of the $N_2$ subunit peptide CNBr-2 and those of the proteins encoded by clones $GTH_4$ and GST mu2. At position 66 the $N_2$ subunit contains threonine whereas the proteins encoded by clones $GTH_4$ and GST mu2 contain alanine, and at position 77 the $N_2$ subunit possesses arginine whereas the proteins encoded by clones $GTH_4$ and GST mu2 both contain cysteine. However, when the sequence of the $N_2$-subunit peptide CNBr-2 is compared with that of the protein encoded by clone GST mu3 it is apparent that the two sequences correspond exactly over the region where sequence data on this genomic clone are available (i.e. residues 37-78).

Residues 133-153 of subunit $N_2$ were identified by sequencing the peptide, CNBr-5, that was eluted at 51 min from the $\mu$ Bondapak $C_{18}$ column (peak d). Five amino acid substitutions were present between the sequence of $N_2$-subunit peptide CNBr-5 and that of the protein encoded by clone $GTH_4$. At position 133 a methionine residue, which has provided a CNBr-cleavage site, can be assigned to subunit $N_2$, whereas the protein encoded by clone $GTH_4$ contains a lysine residue. The other four amino acid changes between the $N_2$-subunit peptide CNBr-5 and the protein encoded by clone $GTH_4$, at positions 139, 144, 148 and 150, were as follows: glutamine to glutamic acid, glutamine to arginine, leucine to alanine and aspartic acid to asparagine, respectively. The $N_2$-subunit peptides CNBr-6 and CNBr-7, which were eluted at 30 min and 23 min from the reverse-phase column (peaks b and a), contained residues 198-210 and residues 212-217 respectively. No differences were apparent between this C-terminal region of the $N_2$ subunit and the homologous region of the protein encoded by clone $GTH_4$.

Cyanogen bromide cleavage of GST $N_3N_3$ permitted the sequencing of one peptide (CNBr-6), which eluted at 41.5 min from the μ Bondapak $C_{18}$ column, represented by peak a on the chromatogram shown in Figure 8.07. The homologous region of the protein encoded by clone $GTH_4$ (residues 184-204), together with a sequence deduced from the cDNA clone HTGT-6 (Campbell *et al.*, 1990) are shown aligned for comparison with the amino acid sequence of the $N_3$-subunit peptide (Figure 8.09). Of the fourteen residues from the $N_3$-subunit peptide CNBr-6, which could be identified with confidence, only six were identical to those of the protein encoded by clone $GTH_4$. The eight amino acid changes between the $N_3$-subunit peptide CNBr-6 and the protein encoded by clone $GTH_4$ were as follows: methionine to isoleucine, alanine to serine, leucine to methionine, glutamine to lysine, aspartic acid to serine, glutamine to arginine, cysteine to leucine and lysine to proline. However, when the sequence of the $N_3$-subunit peptide CNBr-6 is compared with that of the protein encoded by clone HTGT-6 it is apparent that the two sequences correspond exactly.


**Figure 8.09 Comparison of a part of the primary structure of the $N_3$ subunit with the sequences of other human Mu-class GST**

The amino acid sequence of a CNBr-derived peptide (CNBr-6) from subunit $N_3$ is shown aligned for comparison with the homologous sequences deduced from the cDNA $GTH_4$ (DeJong *et al.*, 1988) and the cDNA HTGT-6 (Campbell *et al.*, 1990). Residues are numbered according to the amino acid sequence deduced from cDNA HTGT-6, which encodes four more amino acid residues, than cDNA $GTH_4$, at the N-terminus. An asterisk (*) indicates amino acid residues that differ from those in CNBr-6. The putative methionine residue is in parentheses and a dash indicates a position where no residue could be identified with confidence.

# Chapter 9 Purification of human hepatic Theta-class GST

## 9.01 A fourth cytosolic class of GST

Recent reports have described cytosolic GST isoenzymes which are structurally unrelated to the Alpha, Mu and Pi classes of GST (Hiratsuka *et al.*, 1990; Meyer *et al.*, 1991). Following the purification of two isoenzymes from rat liver (GST 5-5 and 12-12) and one from human liver (GST *θ*) Meyer *et al.* (1991) have proposed that these isoenzymes comprise a fourth class of cytosolic GST which should be designated Theta. The Theta-class GST differ from the GST of the Alpha-, Mu- and Pi-classes in that they lack catalytic activity towards the commonly used GST substrate 1-chloro-2,4-dinitrobenzene and are not retained by the S-hexylglutathione and glutathione affinity matrixes. Substrates that have been used to identify Theta-class GST and monitor them during purification include 1,2-epoxy-3-(*p*-nitrophenoxy)propane and 5-hydroxymethylchrysene sulphate.

Prior to the description of Theta-class GST by Meyer *et al.* (1991) several novel rat liver GST which catalyse the inactivation of reactive sulphate esters were identified by Hiratsuka *et al.* (1990). One of these isoenzymes, a homodimeric protein called GST YrsYrs, was purified from rat liver cytosol and like GST 5-5 and 12-12 it was shown to be structurally and immunologically distinct from Alpha-, Mu- and Pi-class GST. GST YrsYrs has N-terminal amino acid sequence homology with the Theta-class GST described by Meyer *et al.* (1991), however, unlike these enzymes, it does not possess GST activity towards 1,2-epoxy-3-(*p*-nitrophenoxy)propane. The compound 1-menaphthyl sulphate was found to be the best substrate for GST YrsYrs among the arylmethyl sulphates examined by these workers.

In this thesis the expression of Theta-class isoenzymes in human liver have been investigated using activity with the substrate 1-menaphthyl sulphate as a means of establishing the identity of these GST. The purification of a human hepatic GST active towards this sulphate ester will be presented in this chapter.

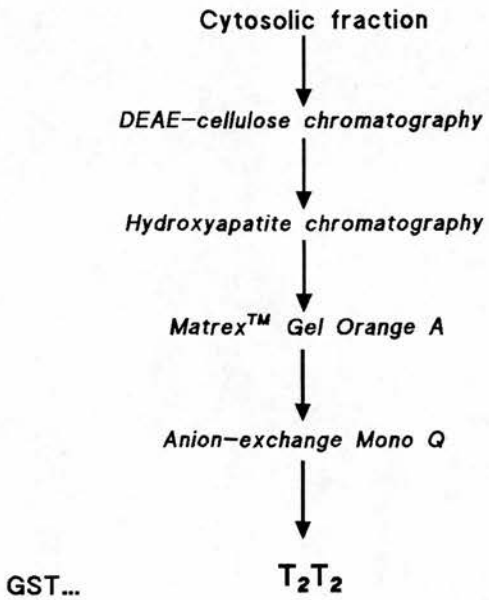
## 9.02 Purification strategy for human hepatic Theta-class GST

The purification protocol adopted for the isolation of a human Theta-class GST, designated $T_2T_2$, is depicted in Scheme 9.01. Preliminary experiments revealed that the chromatographic behaviour of this human enzyme was distinct from that described for either rat GST YrsYrs (Hiratsuka *et al.*, 1990) or the Theta-class GST purified by Meyer *et al.* (1991). Unlike GST YrsYrs, the GST activity towards 1-menaphthyl sulphate present in a human liver sample was not retained by the dye-ligand Blue Sepharose. However, Matrex™ Gel Orange A, a chromatographic gel used during the purification of rat GST 5-5 and 12-12 and human GST $\theta$ (Meyer *et al.*, 1991), was found to bind GST $T_2T_2$. The behaviour of GST $T_2T_2$ on anion-exchange chromatography indicated that it was a more acidic protein than either GST 5-5, 12-12 or $\theta$ , in that it was retained on Mono Q at pH 8.0, whereas a higher pH (9.55) was necessary to retain these Theta-class GST. The 1-menaphthyl sulphate activity from a human liver sample was also retained during chromatofocusing on a column containing polybuffer exchanger PBE 94 at pH 7.0. Thus indicating that the pI of GST $T_2T_2$, as estimated by chromatofocusing, is $\leq$ 7.0.

The purification of GST $T_2T_2$ was achieved in four chromatographic steps. Anion-exchange on DEAE-cellulose was followed by hydroxyapatite chromatography, a technique which successfully resolves the human hepatic Alpha-, Mu- and Pi-class GST. The third step involved chromatography on Matrex™ Gel Orange A, which bound a large proportion of the GST activity towards 1-menaphthyl sulphate present in the human liver sample and resulted in a significant increase in specific activity. Finally, chromatography on Mono Q yielded a homogeneous preparation of GST $T_2T_2$.

## 9.03 Purification of a Theta-class GST from human liver

A cytosolic fraction was prepared from 624 g of human liver as described above (see sections 4.02 and 4.10). The specific activity of human liver cytosol with 1-menaphthyl sulphate as substrate was found to reduce significantly with storage at 4 °C. Immediately following preparation, the specific activity of the cytosol for 1-menaphthyl sulphate was 1.39 ± 0.08 nmol/min per mg of protein, whereas, after 24 h at 4 °C a specific activity of 0.29 ± 0.04 nmol/min per mg was recorded. Because of this dramatic loss of activity the sample was applied immediately, without prior dialysis, to two columns containing DEAE-cellulose. These two columns were developed in parallel and the elution profiles obtained, one of which is shown in Figure 9.01, were closely similar. A proportion of the




**Scheme 9.01 Purification of human Theta-class GST**

**Figure 9.01 Elution of human liver cytosolic GST from DEAE-cellulose**

Anion-exchange chromatography on DEAE-cellulose was performed at pH 8.25 in 1
Tris/HCl buffer, containing 1 mM EDTA and 0.5 mM dithiothreitol, as described in s
4.04e. Human liver cytosol (specimen L5) was prepared and immediately appl
columns (4.4 cm x 90 cm), containing DE-52. Following a wash with approx. 100
the equilibration buffer a 0-0.3M NaCl gradient (▪) was used to develop the
exchanger. The resulting fractions (11.5 ml) were assayed for GST activity towa
menaphthyl sulphate (●) and 1-chloro-2,4-dinitrobenzene (▲) and the absorbance
nm (○) was measured. Those fractions which were combined for further purificati
indicated by the horizontal bar.

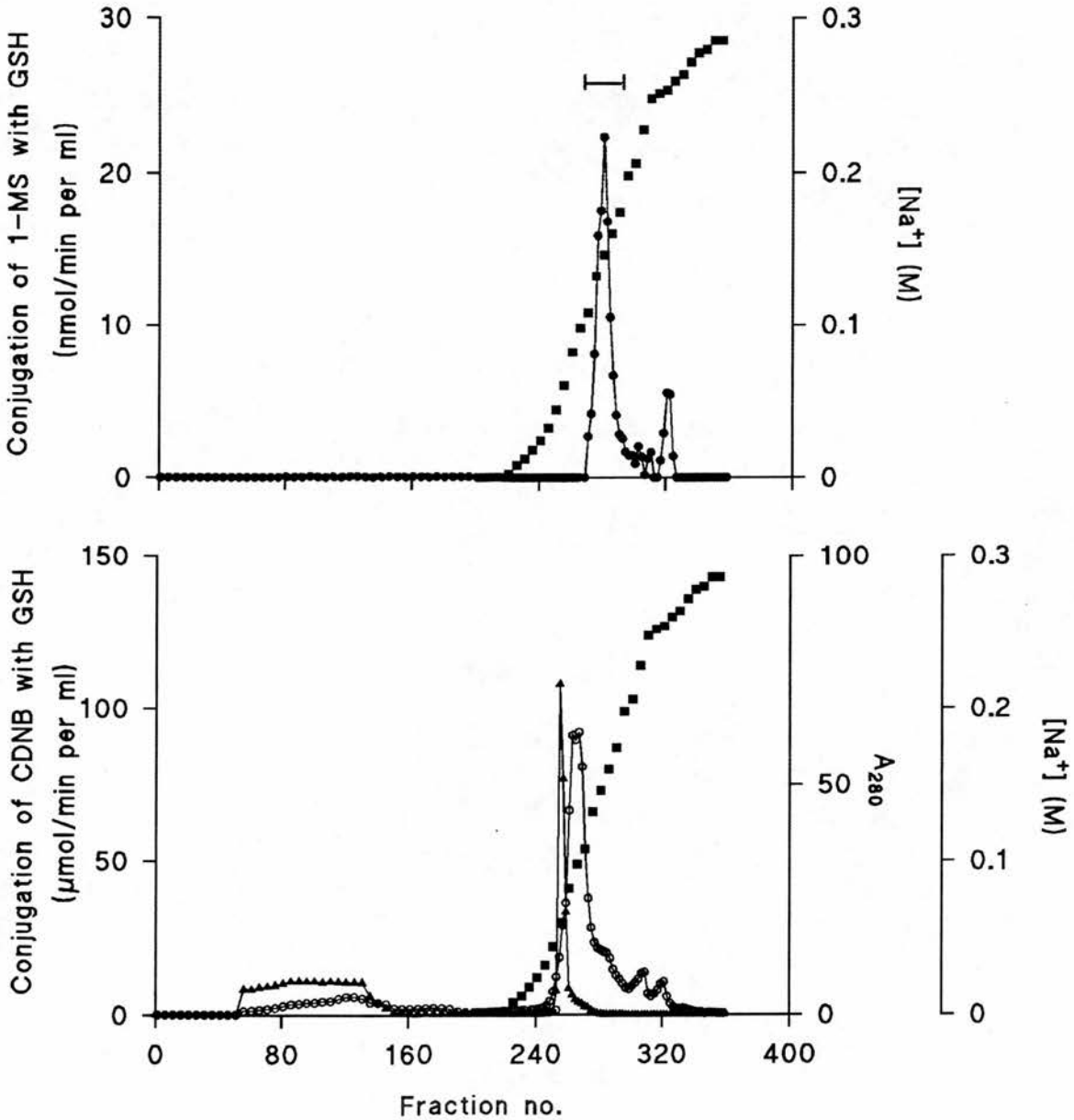

Conjugation of 1-MS with GSH
(nmol/min per ml)
30
20
10
0
0.3
0.2
0.1
0
[Na+] (M)
Conjugation of CDNB with GSH
(μmol/min per ml)
150
100
50
0
100
50
0
A280
0.3
0.2
0.1
0
[Na+] (M)
0
80
160
240
320
400
Fraction no.

**Figure 9.01**

GST activity with 1-chloro-2,4-dinitrobenzene as substrate was not retained by the column and the remainder eluted as a peak on the salt gradient between 40-60 mM NaCl. A peak of GST activity towards 1-menaphthyl sulphate, which did not co-elute with the peak of activity towards 1-chloro-2,4-dinitrobenzene, was resolved between 110-160 mM NaCl on the salt gradient. A second minor peak of activity towards 1-menaphthyl sulphate, which was not retained for further purification, eluted on the gradient at 250-280 mM NaCl.

The fractions collected following hydroxyapatite chromatography of the DEAE-cellulose purified material were assayed for glutathione peroxidase activity with cumene hydroperoxide as substrate as well as GST activity towards 1-menaphthyl sulphate and 1-chloro-2,4-dinitrobenzene (Figure 9.02). The GST activity towards 1-menaphthyl sulphate eluted as two peaks on the sodium phosphate gradient; one between 40-70 mM sodium phosphate and a second between 130-170 mM sodium phosphate. Both of these peaks contained glutathione peroxidase activity. Those fractions in the first peak of GST activity towards 1-menaphthyl sulphate (i.e. which eluted at 40-70 mM sodium phosphate) that were not coincident with activity towards 1-chloro-2,4-dinitrobenzene were pooled for further purification, as shown in Figure 9.02.

The third purification step involved chromatography on Matrex™ Gel Orange A (Figure 9.03). Approximately one third of the GST activity towards 1-menaphthyl sulphate was not retained by this column. However, the remaining GST activity towards 1-menaphthyl sulphate eluted as a single peak, between 0.2-0.3 M KCl on the gradient, that was well resolved from the GST activity towards 1-chloro-2,4-dinitrobenzene. Those fractions active towards 1-menaphthyl sulphate were also found to contain glutathione peroxidase activity.

The material which was active toward 1-menaphthyl sulphate, that was resolved on the KCl gradient following Matrex™ Gel Orange A chromatography was subjected to a final chromatographic step on Mono Q f.p.l.c. (Figure 9.04a). The GST activity towards 1-menaphthyl sulphate and the glutathione peroxidase activity towards cumene hydroperoxide eluted as a single peak between 80-90 mM NaCl on the salt gradient.

### 9.04 Purity and subunit analysis of human Theta-class GST $T_2T_2$

SDS/PAGE analysis of the fractions from Mono Q revealed that the peak which was active with 1-menaphthyl sulphate contained a single polypeptide band of subunit $M_r$ 25100

**Figure 9.02 Hydroxyapatite chromatography of human hepatic GST**

Material from the DEAE-cellulose column was subjected to chromatography on a c
(3.3 cm x 45 cm) containing hydroxyapatite equilibrated with 10 mM sodium phos
buffer, pH 7.0, that contained 1 mM EDTA and 0.5 mM dithiothreitol, as descril
section 4.04c. A 10-250 mM sodium phosphate gradient (▪) was used to develo
column and the eluate was monitored for GST activity towards 1-menaphthyl sulpha
and 1-chloro-2,4-dinitrobenzene (▲), glutathione peroxidase activity towards cu
hydroperoxide (△) and absorbance at 280 nm (○). The horizontal bar indicates the
fractions which were pooled for further purification.

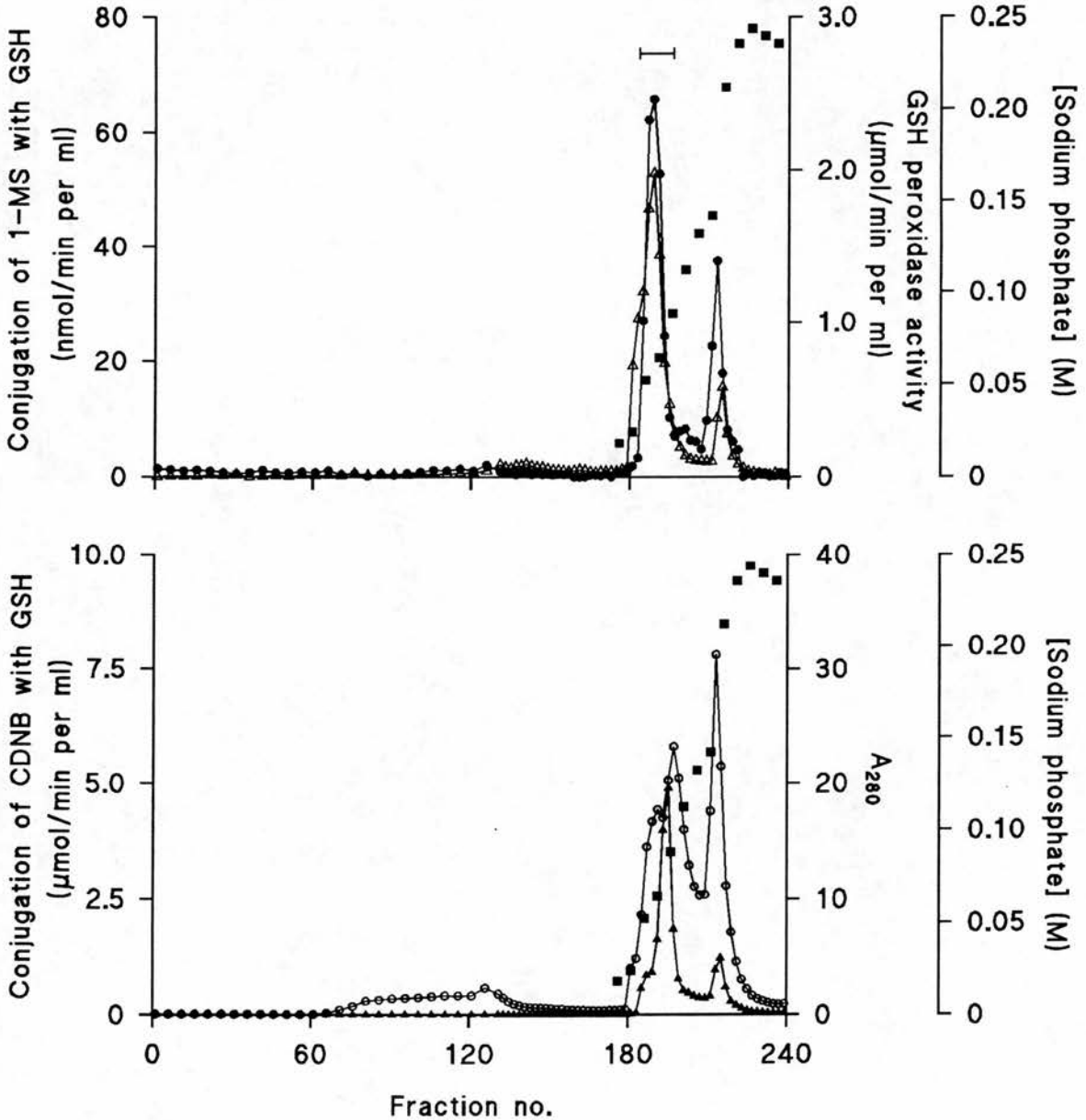

Conjugation of 1-MS with GSH
(nmol/min per ml)
GSH peroxidase activity
(μmol/min per ml)
[Sodium phosphate] (M)
Conjugation of CDNB with GSH
(μmol/min per ml)
A280
[Sodium phosphate] (M)
Fraction no.

**Figure 9.02**

**Figure 9.03 Dye-ligand chromatography of human hepatic Theta-class GST**

The preparation of human Theta-class GST recovered from hydroxyapatite was subj
to chromatography on a column (1.6 cm x 26 cm) containing Matrex™ Gel Oran
equilibrated with 10 mM sodium phosphate buffer, pH 6.8, containing 1 mM EDTA an
mM dithiothreitol. The sample was dialysed extensively against the equilibration b
before application to the column as described in section 4.04b. Following the form
of a 0-1.0 M KCl gradient (▪), in the equilibration buffer, the fractions (3.4 ml) coll
were analysed for GST activity using 1-menaphthyl sulphate (●) and 1-chloro-2,4-di
benzene (▲) as substrates, glutathione peroxidase activity using cumene hydroper
(△) as substrate and absorbance at 280 nm (○). The peak of GST activity towar
menaphthyl sulphate which was resolved on the salt gradient was pooled as indicat
the horizontal bar.

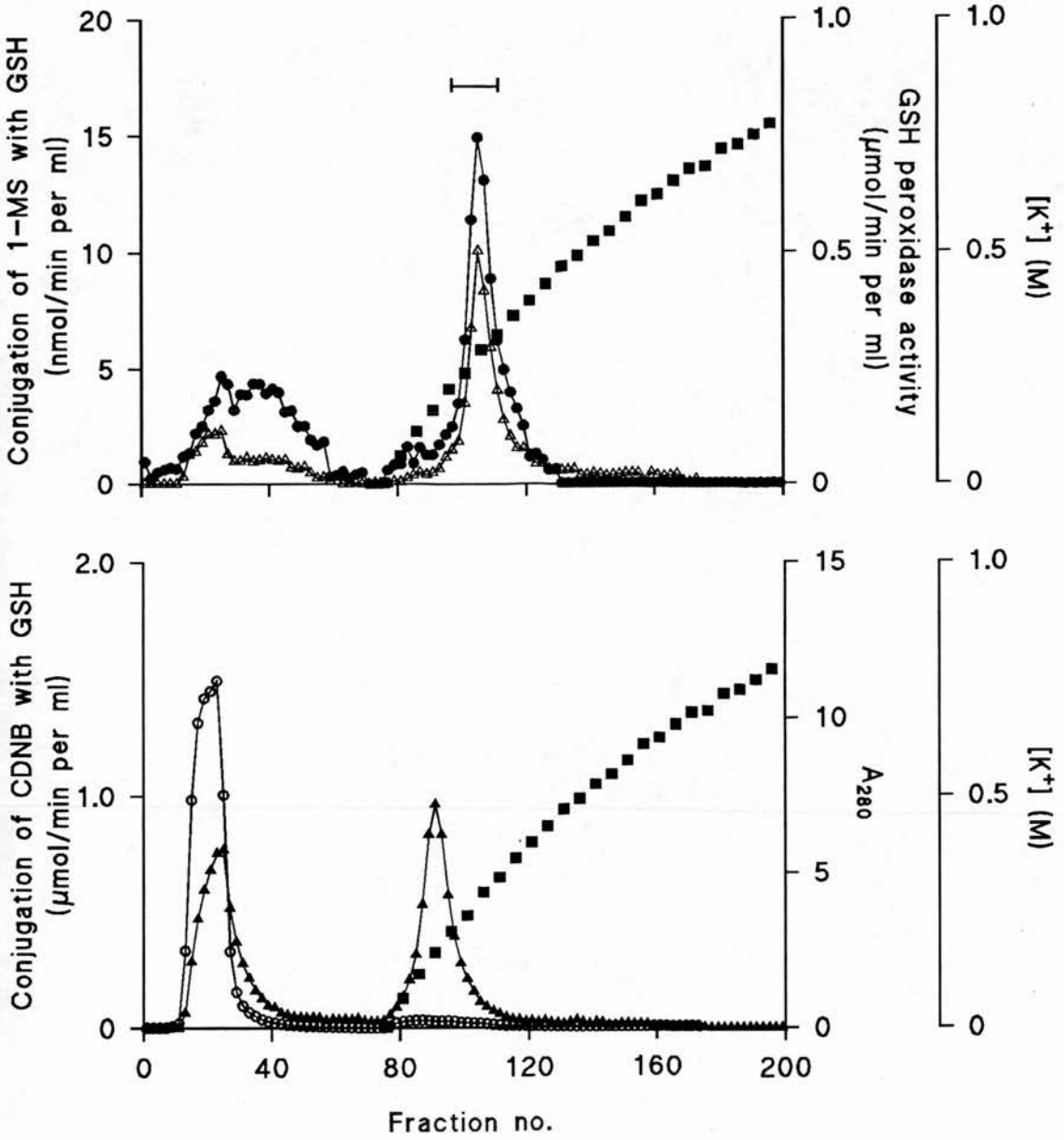

Conjugation of 1-MS with GSH
(nmol/min per ml)
GSH peroxidase activity
(μmol/min per ml)
[K+] (M)
Conjugation of CDNB with GSH
(μmol/min per ml)
A280
Fraction no.

**Figure 9.03**

**Figure 9.04 Purification of a human hepatic Theta-class GST by anion-exc f.p.l.c. and SDS/PAGE analysis of combined fractions.**

The material purified by DEAE-cellulose, hydroxyapatite and dye-ligand chromatog was dialysed extensively against 20 mM Tris/HCl buffer, pH 8.4 at 4 °C, containing C dithiothreitol, before application to Mono Q f.p.l.c. as described in section 4.06a (pa The column was eluted at 0.75 ml/min and developed with a 0-1.0 M NaCl gradient dialysis buffer; this gradient was established in two steps as shown by the straigl Fractions (0.75 ml) were collected and the absorbance at 280 nm (___) was mon Those fractions which contained GST activity towards 1-menaphthyl sulphate were p as indicated by the horizontal bar, and subjected to SDS/PAGE analysis (panel b). 1, 2 and 3 were loaded with the human subunit markers GST $B_1B_2$ ($M_r$ 26000), GS 26700) and GST $\pi$ ($M_r$ 24800), respectively. Track 4 contained the pool ob following Mono Q f.p.l.c. (2 $\mu$g).

(a)

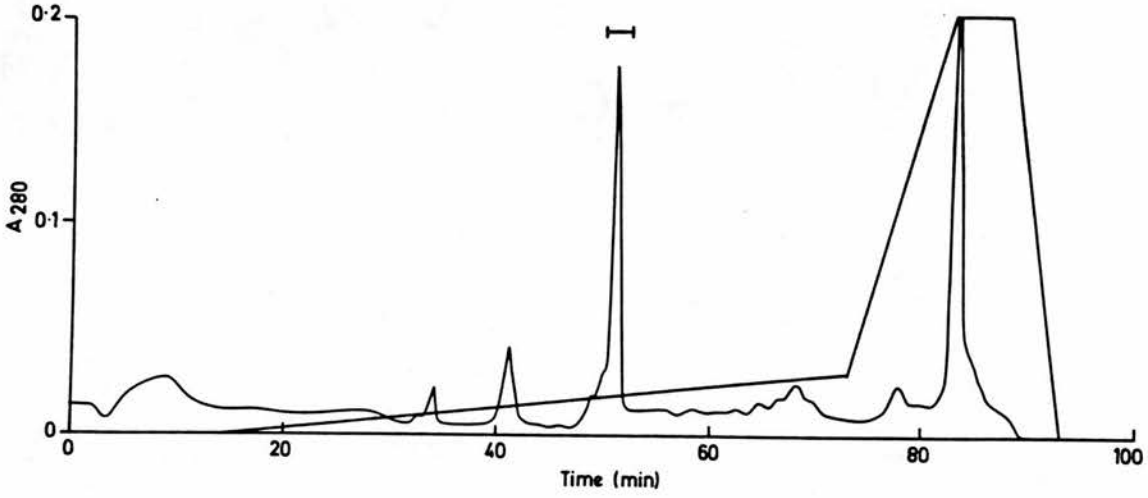



**Figure 9.04**

(b)

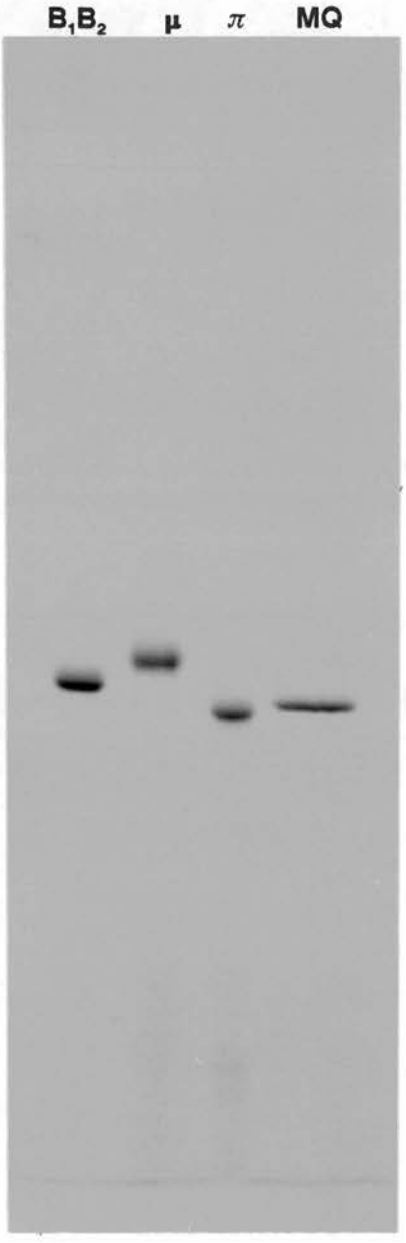



**Figure 9.04**

(Figure 9.04b) indicating that the enzyme comprised subunits of equal size. The results of the purification of this transferase are shown in Table 9.01.

This GST was judged to be 95% pure when analysed by reverse-phase h.p.l.c. (Figure 9.05). Human Alpha-class GST, which were present in an S-hexylglutathione affinity-purified pool, were applied to the reverse-phase column as a means of calibration; subunits $B_1$ and $B_2$ eluted from the μ Bondapak $C_{18}$ column at 50 and 56 min respectively. The GST with activity for 1-menaphthyl sulphate eluted from the reverse-phase column as a single symmetrical peak at 62 min. As these data suggest that this Theta-class enzyme is a homodimeric protein containing a single subunit type it has been designated GST $T_2T_2$.

**Table 9.01 Purification of a human hepatic Theta-class GST**

For experimental details see section 4.10. GST activity was measured with 1-menaphthyl sulphate at 37 °C.

| Fraction | Total protein (mg) | Total activity (nmol/min) | Specific activity (nmol/min/mg) | Recovery (%) |
|---|---|---|---|---|
| Cytosol | 38160 | 53136 ± 2988 | 1.39 ± 0.08 | 100 |
| Cytosol following 24 h at 4 °C | 38160 | 11178 ± 1548 | 0.29 ± 0.04 | 21 |
| DEAE-cellulose pool | 4030 | 5304 ± 377 | 1.32 ± 0.09 | 9.9 |
| Hydroxyapatite pool | 949.5 | 1723.5 ± 23.4 | 1.82 ± 0.02 | 3.2 |
| Matrex™ Gel Orange A pool | 3.68 | 258 ± 7.5 | 70.1 ± 2.04 | 0.5 |
| Mono Q f.p.l.c. pool | 0.195 | 96.9 ± 3.77 | 497.2 ± 19.4 | 0.17 |

**Figure 9.05 Preparative reverse-phase h.p.l.c. of human Theta-class GST**

A portion (60 $\mu$g) of human hepatic GST $T_2T_2$, purified by sequential DEAE-ce chromatography, hydroxyapatite chromatography, Matrex™ Gel Orange A chromato and Mono Q f.p.l.c., was subjected to reverse-phase h.p.l.c. on a Waters µ Bondap column (10 $\mu$m particle size; 0.39 cm x 30 cm). The column was developed by a 40-55% (v/v) acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid formed over that was followed by a 55-70% (v/v) acetonitrile gradient in aq. 0.1% (v/v) trifluor acid formed over 5 min. The eluate was monitored continuously at 220 nm (___

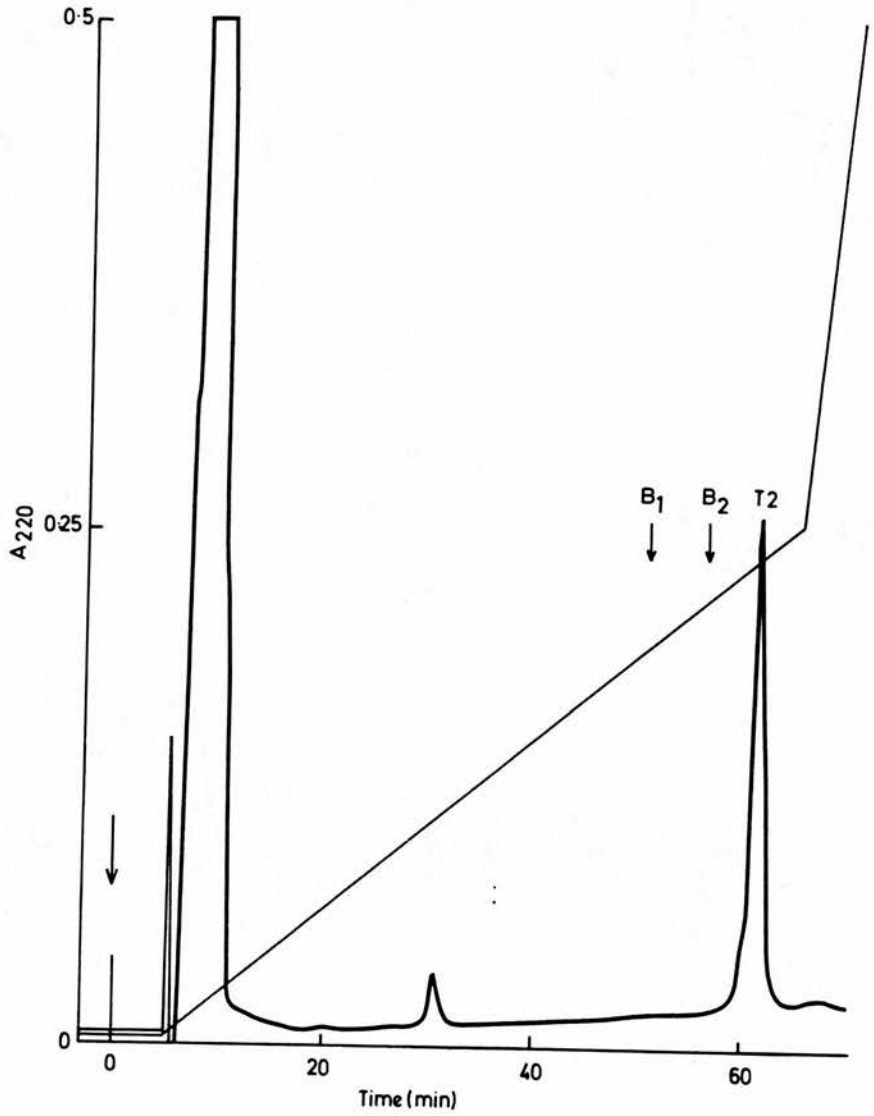



**Figure 9.05**

# Chapter 10 Purification of Theta-class GST from mouse liver

The cytosolic fraction from the livers of mice fed normal diets contains GST isoenzymes which are representative of the Alpha-, Mu- and Pi-classes; GST $Ya_3Ya_3$, $Yb_1Yb_1$ and YfYf respectively (McLellan and Hayes, 1989). To date, the existence of isoenzymes which are unrelated to any of these three well characterized cytosolic classes of GST has not been documented in the mouse.

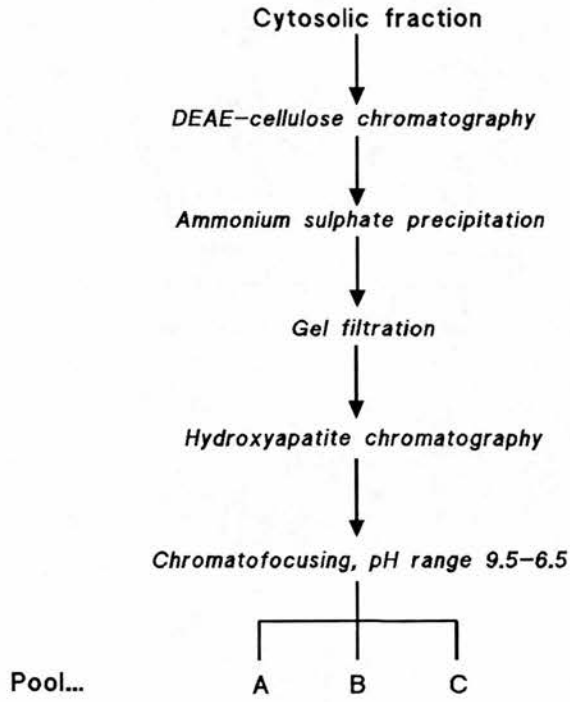
In this thesis, the expression of hepatic murine GST active towards the substrate 1-menaphthyl sulphate has been investigated, to enable a comparison between rodent Theta-class forms and the human liver Theta-class GST designated $T_2T_2$.

## 10.01 Strategy for the purification of murine Theta-class GST

An adaptation of the protocol by Hiratsuka *et al.* (1990), for the isolation of rat GST YrsYrs, was used for the purification of mouse GST (Scheme 10.01). Murine GST, active towards 1-menaphthyl sulphate, behaved in a similar manner to GST YrsYrs during DEAE-cellulose chromatography and chromatofocusing. The mouse GST, like GST YrsYrs, was retained by the dye-ligand Blue Sepharose. However, this step was omitted from the purification scheme because a substantial loss of 1-menaphthyl sulphate conjugating activity (approx. 85%) was observed when it was used in a preliminary study.

## 10.02 Purification of mouse Theta-class GST

A cytosolic fraction was prepared from the livers of male C57BL6 mice. Following dialysis, at 4 °C for 24 h, the specific activity of the cytosol, towards 1-menaphthyl sulphate, was 1.56 ± 0.02 nmol/min per mg of protein. All of this activity was retained on DEAE-cellulose at pH 8.25 and eluted as a single peak between 30-70 mM NaCl on the gradient (Figure 10.01). The majority of the GST activity towards 1-chloro-2,4-dinitrobenzene, on the other hand, did not bind to the column and was eluted before the gradient was developed.




**Scheme 10.01** **Purification of murine Theta-class GST**

**Figure 10.01 DEAE-cellulose chromatography of murine hepatic GST**

Hepatic cytosol, prepared from the livers of C57BL6 adult male mice, was dialysed aga two changes, each of 5 litres, of 10 mM Tris/HCl buffer, pH 8.25, containing 1 mM E and 2 mM 2-mercaptoethanol, before application to a column (4.4 cm x 90 cm) contai DE-52 equilibrated in the same buffer. A non-linear 0-0.3 M NaCl gradient (▪), in dialysis buffer, was used to develop the column and the fractions (11.5 ml) collected analysed for GST activity towards 1-menaphthyl sulphate (●) and 1-chloro-2,4-dini benzene (▲). The absorbance at 280 nm (○) was also measured. The fractions w were pooled for further purification are indicated by the horizontal bar.

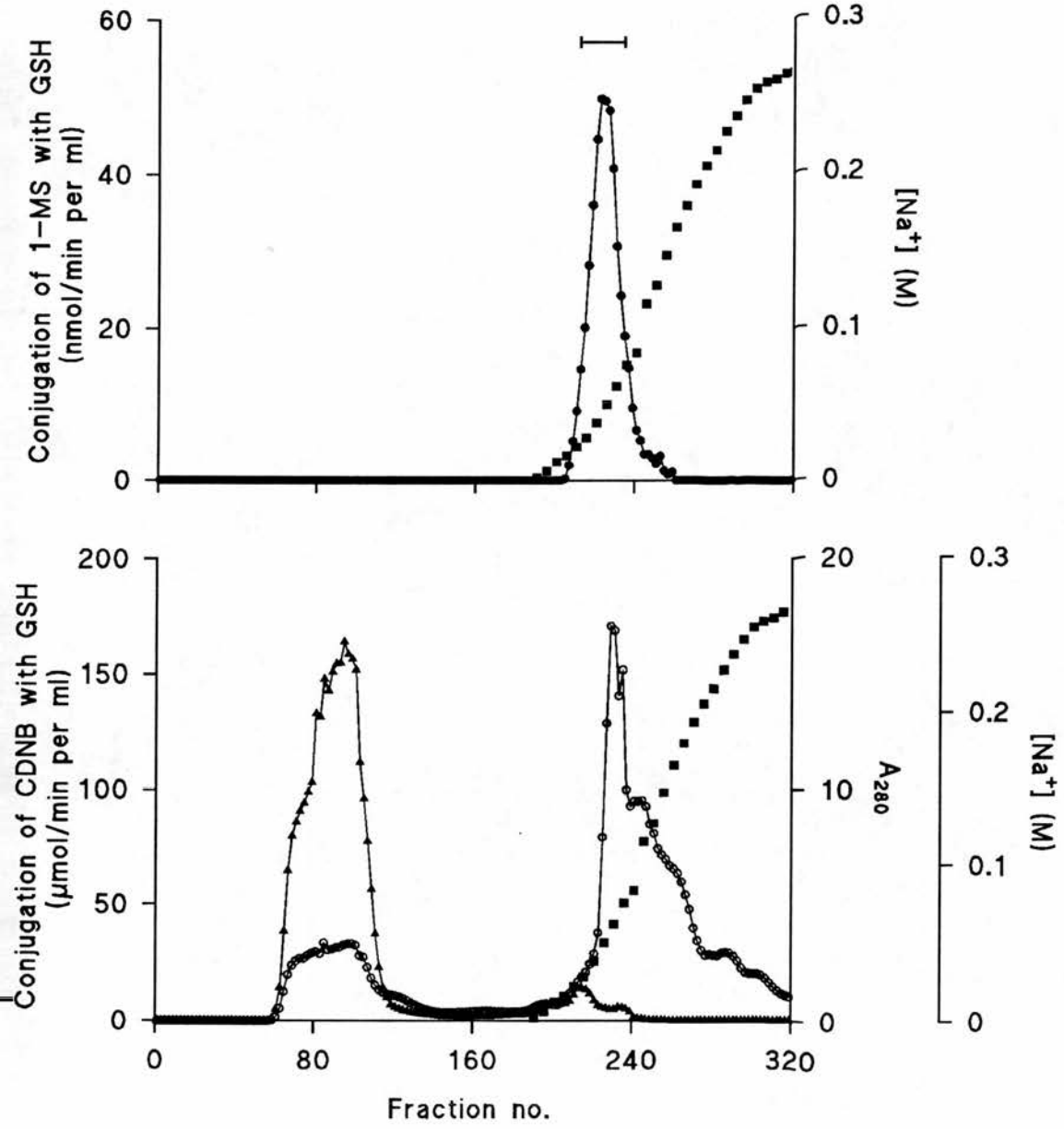

Conjugation of 1-MS with GSH (nmol/min per ml)
60
40
20
0
0.3
0.2
0.1
0
[Na+] (M)
Conjugation of CDNB with GSH (μmol/min per ml)
200
150
100
50
0
20
10
0
A280
0.3
0.2
0.1
0
[Na+] (M)
0
80
160
240
320
Fraction no.

**Figure 10.01**

The protein present in the material isolated by anion-exchange chromatography was treated with ammonium sulphate and the protein precipitated between 30-90% saturation was collected. Gel filtration, on Sephadex G-100, of the concentrated sample resulted in the elution of one peak of GST activity.

The active fractions eluted from the gel filtration column were combined and subjected to hydroxyapatite chromatography. The eluate was monitored for glutathione peroxidase activity with cumene hydroperoxide as substrate as well as transferase activity (Figure 10.02). A single peak of activity towards 1-menaphthyl sulphate, that was coincident with a peak of glutathione peroxidase activity, eluted between 80-120 mM on the sodium phosphate gradient. Two further peaks of glutathione peroxidase activity, which were not coincident with activity towards 1-chloro-2,4-dinitrobenzene, were also resolved on the gradient; one of which possibly represents Se-dependant glutathione peroxidase.

The final chromatofocusing step resolved the 1-menaphthyl sulphate activity recovered from hydroxyapatite into three peaks, none of which had detectable activity either towards 1-chloro-2,4-dinitrobenzene or towards cumene hydroperoxide (Figure 10.03a). The fractions containing each of these three peaks, which eluted at pH's 8.3, 8.15 and 8.0 , were combined separately and designated Pool A, Pool B and Pool C respectively. Electrophoretic analysis revealed that Pool A and Pool B each contained a band of subunit $M_r$ 25400 (Figure 10.03b). Pool C contained the 25400-$M_r$ subunit together with a polypeptide of $M_r$ 27200. The purification of these 1-menaphthyl sulphate active GST pools from mouse liver is shown in Table 10.01.

### 10.03 Analytical reverse phase h.p.l.c. of mouse Theta-class GST subunits

When murine GST Pools A, B and C were applied to the μ Bondapak $C_{18}$ column each resulted in the elution of a peak with a retention time of 52 min (Figure 10.04). Pool C contained an additional peak which eluted at 24 min.

The three GST pools each appear to contain an identical subunit with respect to mobility on SDS/PAGE and retention time on h.p.l.c. It is possible, therefore, that they represent a single homodimeric GST form.

**Figure 10.02 Elution pattern of mouse liver GST from hydroxyapatite**

DEAE-cellulose purified material from mouse cytosol was precipitated by ammo sulphate (section 4.03), and gel-filtered on Sephadex G-100 (section 4.04f) b application to a column (1.6 cm x 30 cm) of hydroxyapatite which had been equilib with 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 2 m mercaptoethanol (section 4.04c). The column was developed with a linear 10-250 sodium phosphate gradient (▪) in the running buffer. Fractions of 2.3 ml were colle and the absorbance at 280 nm (○), GST activity with 1-menaphthyl sulphate (●) ar chloro-2,4-dinitrobenzene (▲) as substrates and glutathione peroxidase activity cumene hydroperoxide (△) as substrate were measured. The horizontal bar indicate fractions which were combined.

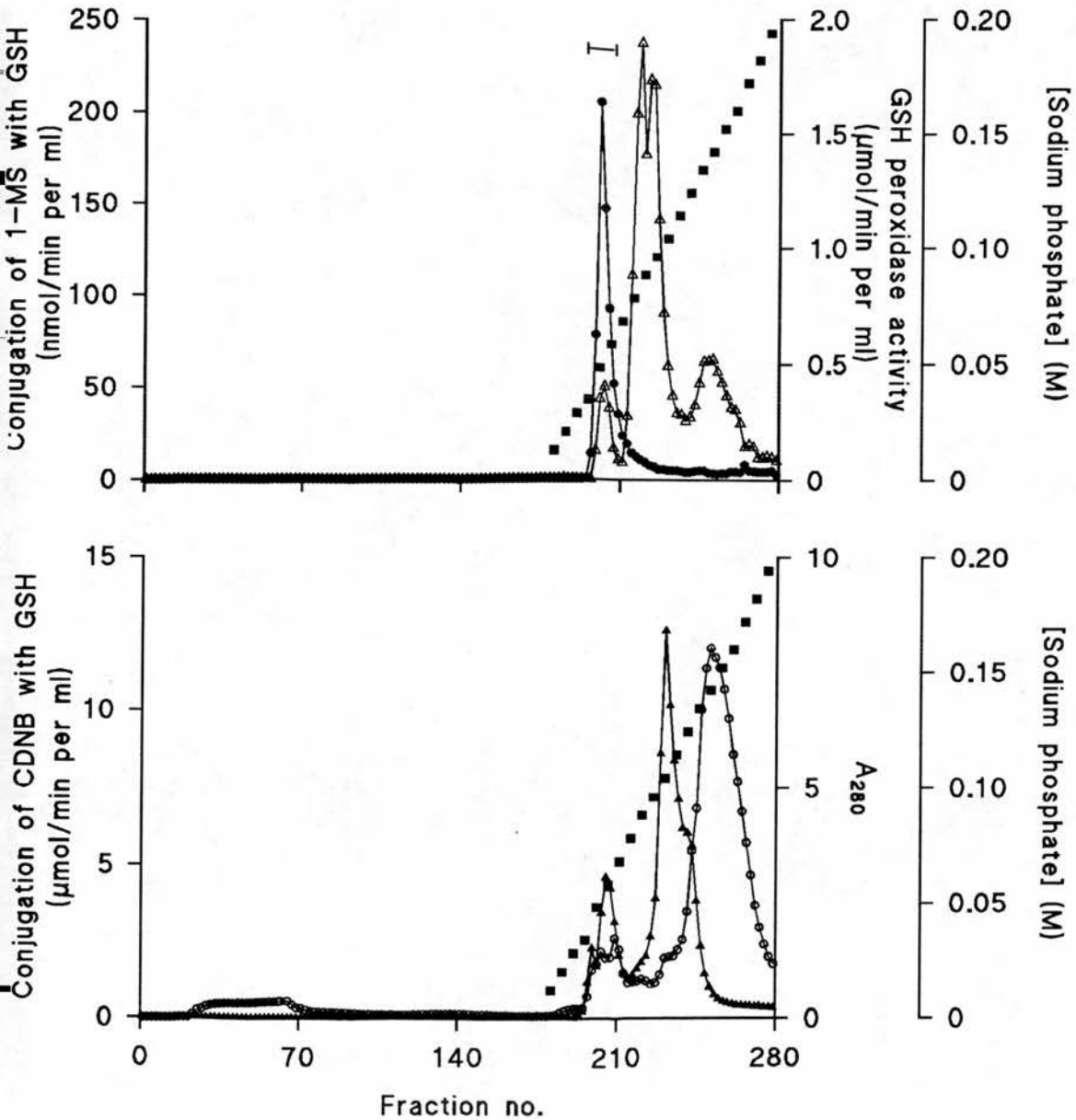

Conjugation of 1-MS with GSH
(nmol/min per ml)
250
200
150
100
50
0
2.0
1.5
1.0
0.5
0
GSH peroxidase activity
(µmol/min per ml)
0.20
0.15
0.10
0.05
0
[Sodium phosphate] (M)
Conjugation of CDNB with GSH
(µmol/min per ml)
15
10
5
0
10
5
0
$A_{280}$
0.20
0.15
0.10
0.05
0
[Sodium phosphate] (M)
0
70
140
210
280
Fraction no.

**Figure 10.02**

**Figure 10.03 Chromatofocusing of murine hepatic Theta-class GST and SDS/P of pooled fractions**

A sample of partially purified murine Theta-class GST, which had been subjecte chromatography on DEAE-cellulose, Sephadex G-100 and hydroxyapatite, was eluted a chromatofocusing column in the pH range 9.5-6.5 as described in section 4.04d (p a). The column (1.6 cm x 30 cm) of polybuffer exchanger PBE 94 was equilibrated 25 mM ethanolamine/HCl buffer, pH 9.5, containing 2 mM 2-mercaptoethanol. Follo sample application, a gradient was formed using polybuffer 96 at pH 6.5. GST ac towards 1-menaphthyl sulphate (●) and 1-chloro-2,4-dinitrobenzene (▲), protein conce tion (○) and the pH at 4 °C (■) were measured in the fractions (3.4) collected. The peaks of 1-menaphthyl sulphate activity were pooled, as indicated by the horizontal and analysed by SDS/PAGE (panel b). Tracks 1 and 5 both contained affinity-purifie liver GST [Ya ($M_r$ 25500), Yb ($M_r$ 26300) and Yc ($M_r$ 27500)]. Tracks 2, 3 and 4 loaded with Pool A, Pool B and Pool C respectively (approx. 3 $\mu$g of each).

(a)

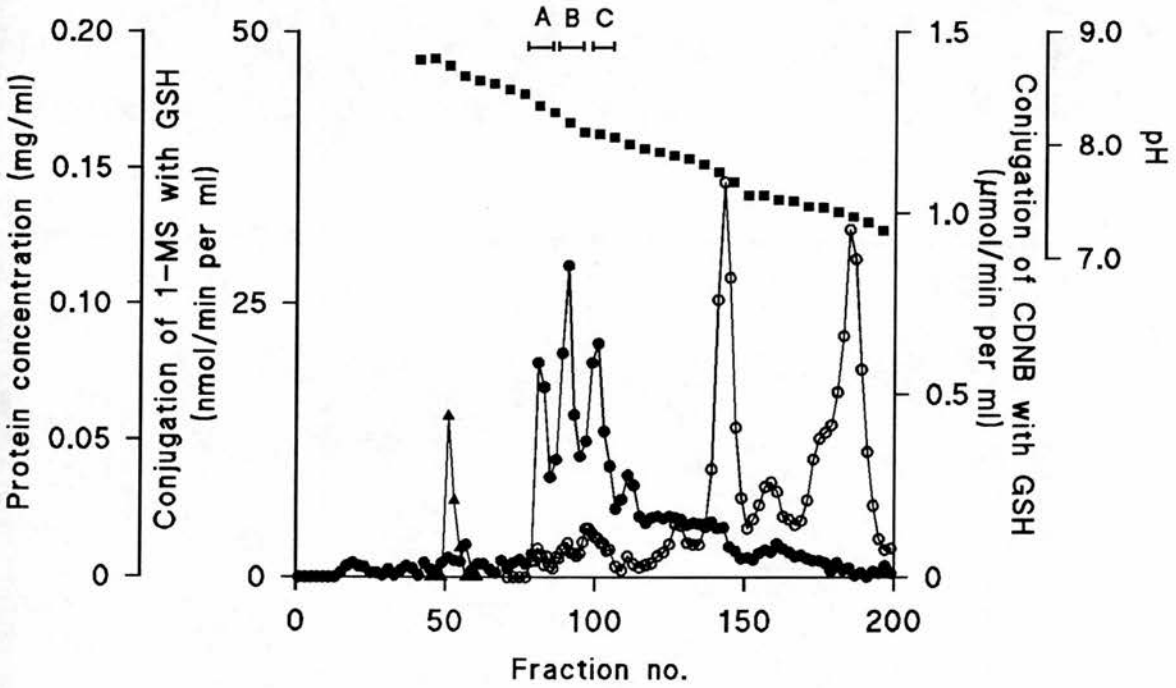



**Figure 10.03**

(b)

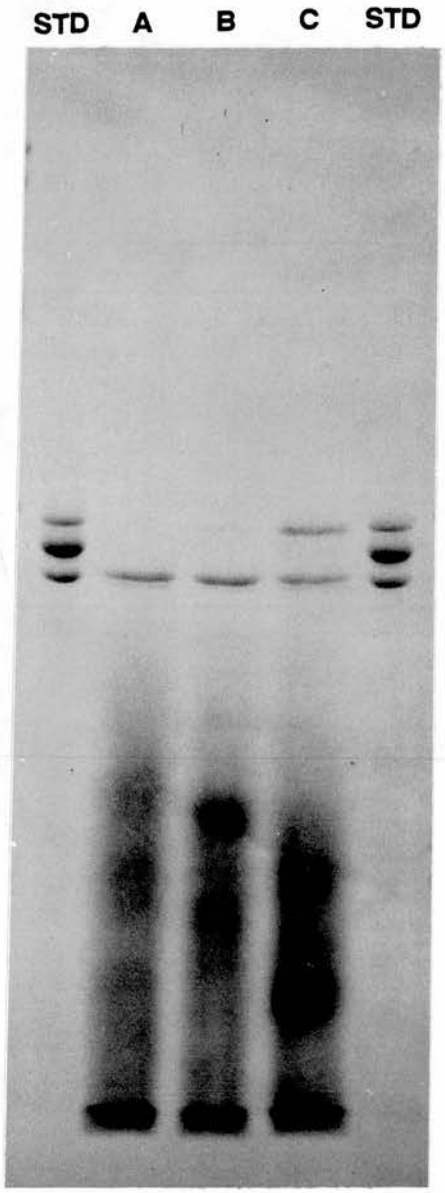



**Figure 10.03**

**Table 10.01 Purification of Theta-class GST from mouse liver**

For experimental details see section 4.11. GST activity was measured with 1-menaphthyl sulphate at 37 °C.

| Fraction | Total protein (mg) | Total activity (nmol/min) | Specific activity (nmol/min per mg) | Recovery (%) |
|---|---|---|---|---|
| Cytosol (post-dialysis for 24 h at 4 °C) | 9520 | 14866.5 ± 204 | 1.56 ± 0.02 | 100 |
| DEAE-cellulose pool | 1359.4 | 8054.7 ± 250 | 5.92 ± 0.18 | 54.2 |
| Sephadex G-100 pool | 514 | 7567 ± 190 | 14.7 ± 0.21 | 50.9 |
| Hydroxyapatite pool | 23.2 | 3906 ± 14 | 168 ± 0.6 | 26.3 |
| Chromatofocusing pools: | | | | |
| Pool A | 0.129 | 211.8 ± 6.7 | 1642 ± 51.5 | 1.4 |
| Pool B | 0.146 | 250.8 ± 19.0 | 1718 ± 130 | 1.7 |
| Pool C | 0.165 | 180.5 ± 12.4 | 1094 ± 75.2 | 1.2 |

**Figure 10.04 Analysis of mouse Theta-class GST subunits by reverse-phase h.**

Analytical reverse-phase h.p.l.c. was performed on the murine Theta-class GST which been purified by sequential DEAE-cellulose chromatography, ammonium sulp precipitation, gel filtration, hydroxyapatite chromatography and chromatofocusing. three peaks of GST activity with 1-menaphthyl sulphate as substrate (Pool A, Pool B Pool C), which were resolved by chromatofocusing in the pH range 9.5-6.5, were applied to the μ Bondapak $C_{18}$ column as described in section 4.05b. A 40-70% acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid was used to develop the co in two linear stages as shown by the straight lines. The $A_{220}$ of the column eluate monitored continuously (___).

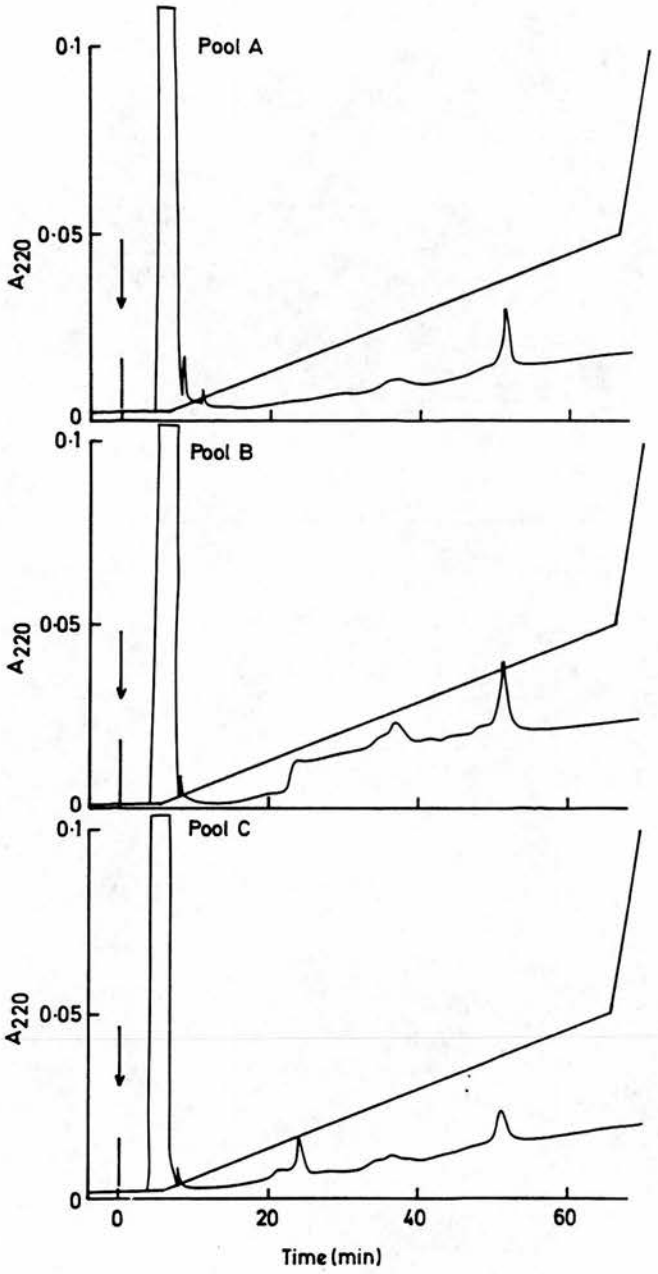



Figure 10.04

# Chapter 11 Characterization of human and murine Theta-class GST

The only human Theta-class isoenzyme which has been described in the literature was isolated on the basis of its activity towards the substrate 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (Meyer *et al.*, 1991). This hepatic isoenzyme, called GST $\theta$, has been partially characterized but its quaternary structure remains to be elucidated.

During the present study a human hepatic enzyme, designated GST $T_2T_2$, and murine hepatic GST have been purified by virtue of their catalytic activity towards the substrate 1-menaphthyl sulphate. The physical, immunochemical, catalytic and structural properties of human GST $T_2T_2$ are presented in this chapter and compared when possible with those of the murine Theta-class GST.

## 11.01 Electrophoretic properties of human and murine Theta-class GST

SDS/PAGE analysis of human GST $T_2T_2$ and the murine GST present in Pool B, obtained following chromatofocusing, revealed that the subunit molecular mass values of these proteins were similar, though not identical (Figure 11.01). $M_r$ values of 25100 and 25400 have been assigned to GST $T_2T_2$ and the subunit present in mouse Pool B, respectively.

## 11.02 Western Blot analysis of human and mouse Theta-class GST

Purified human GST $T_2T_2$ and mouse GST Pool B, together with samples taken following each stage of the purification of the human Theta-class enzyme, were probed with antisera raised against human Alpha-, Mu- and Pi-class GST (Figure 11.02). GST $T_2T_2$ was shown to be immunochemically distinct from other human enzymes of classes Alpha, Mu and Pi, in that no cross-reactivity was observed between this enzyme and antisera raised against either GST $B_1B_1$, GST $\mu$ or GST $\pi$. This analysis also demonstrated that the liver cytosol, from which GST $T_2T_2$ was isolated, expressed Alpha- and Mu-class GST which were removed following chromatography on DEAE-cellulose, as well as a very small quantity of Pi-class GST that was removed following chromatography on Matrex™ Gel

**Figure 11.01 SDS/PAGE analysis of human and murine Theta-class GST**

Electrophoresis was performed in a resolving gel which contained 12% polyacrylamide and 0.32% (w/v) N,N′-methylenebisacrylamide. Human GST su markers $B_1B_2$ ($M_r$ 26000), $\mu$ ($M_r$ 26700) and $\pi$ ($M_r$ 24800) were loaded into tracks 1, 2 3 respectively. Purified human GST $T_2T_2$ (2 $\mu$g) was loaded into track 4. Tra contained murine Theta-class GST (Pool B; 3 $\mu$g).

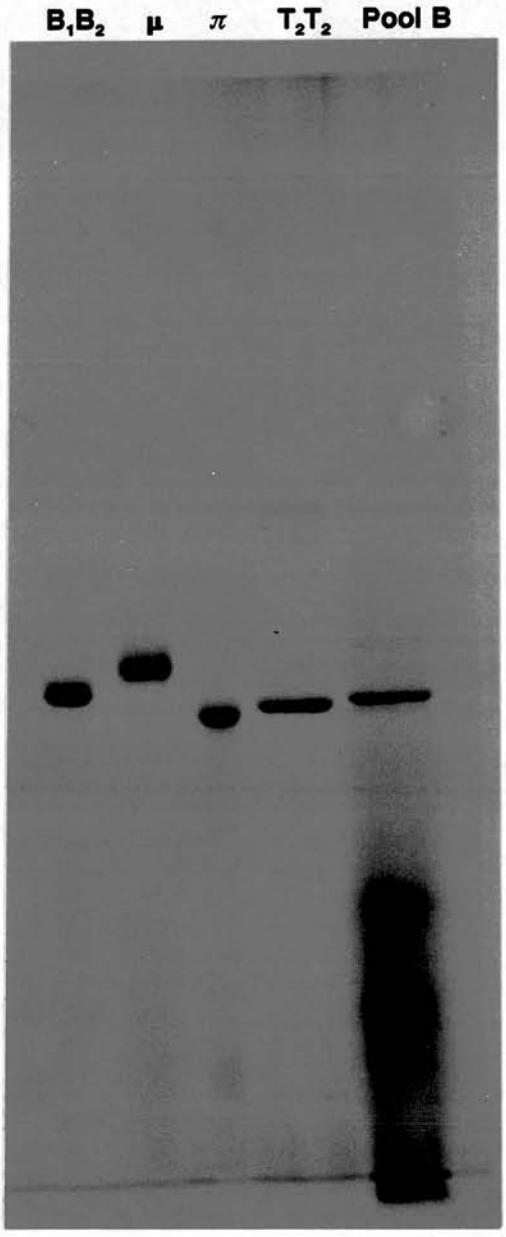



**Figure 11.01**

**Figure 11.02 Cross-reactivity of human and mouse Theta-class GST with ant raised against Alpha-, Mu- and Pi-class GST**

Purified human GST $T_2T_2$, mouse GST Pool B and samples obtained following each of the purification of GST $T_2T_2$ were subjected to immunoblot analysis (Towbin *et al.*, using antisera raised against human Alpha-class GST $B_1B_1$ (panel a), Mu-class G (panel b) and Pi-class GST $\pi$ (panel c). The SDS/polyacrylamide gel was load follows: tracks 1, 2 and 3, human GST $B_1B_1$, $\mu$ and $\pi$ standards; track 4, human cytosol (30 $\mu$g; specimen L5); track 5, DEAE-cellulose pool (30 $\mu$g); track 6, hy apatite pool (30 $\mu$g); track 7, Matrex™ Gel Orange A pool (3 $\mu$g); track 8, purifie $T_2T_2$ obtained following Mono Q f.p.l.c. (2 $\mu$g); track 9 mouse chromatofocusing GS B (2 $\mu$g).

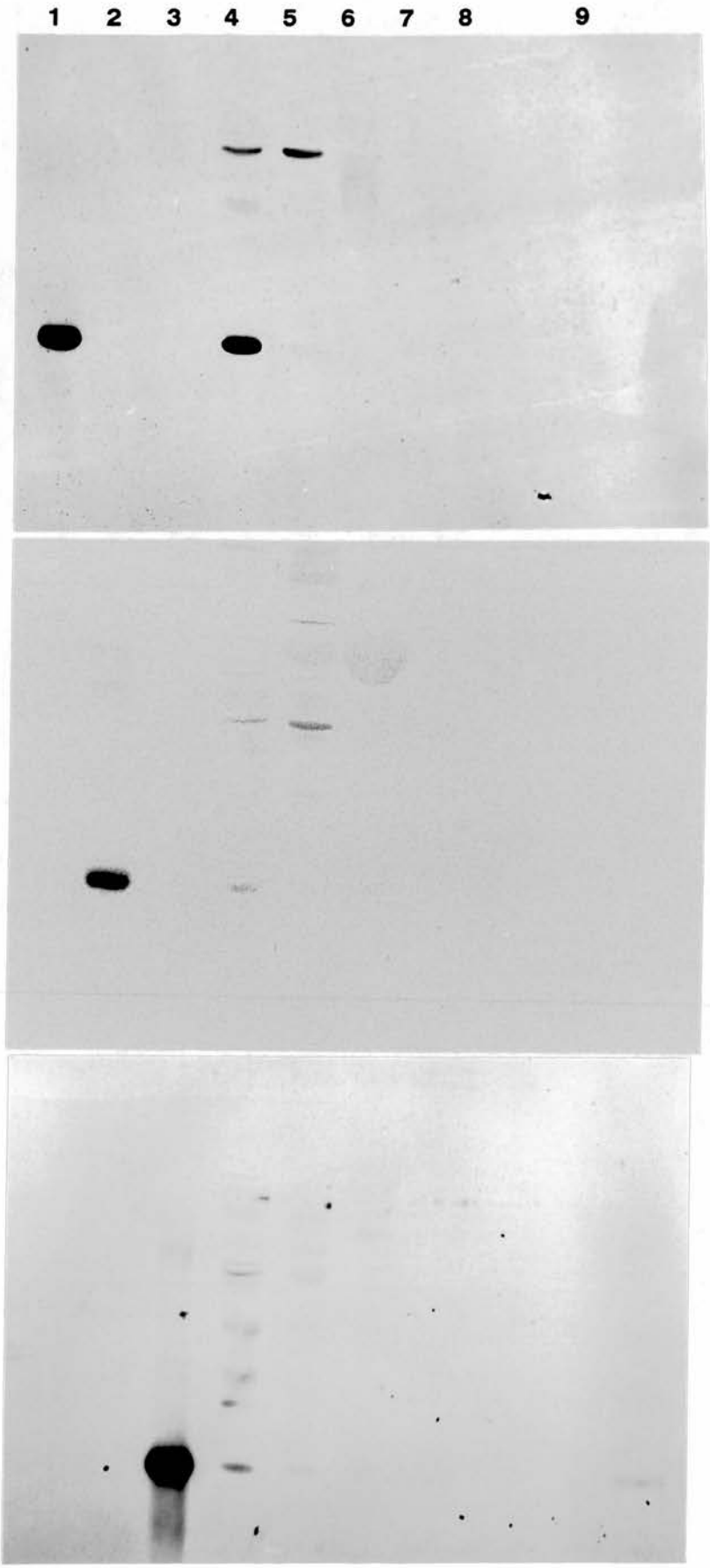



**Figure 11.02**

Orange A. The mouse GST present in Pool B also did not cross-react with antisera raised against either human GST $B_1B_1$ or GST $\mu$. However, a feint cross-reaction was observed with anti-(GST $\pi$) IgG. Whilst this result is surprising it is possible that this represents trace contamination of the Theta-class GST with murine GST YfYf; the Pi-class enzyme is abundant in male mouse liver cytosol, and has a pI value (8.5) close to that at which Pool B eluted from chromatofocusing (McLellan and Hayes, 1987).

The Theta-class GST purified from human and mouse liver were also challenged with an antiserum, provided by Dr. W.B. Jakoby, raised against rat GST E (Figure 11.03). Also included on the Western Blot are; human GST $B_1B_2$, GST $\mu$ and GST $\pi$, rat liver cytosol, as well as samples taken at each stage of the purification of GST $T_2T_2$. The only GST to cross-react with this antiserum were the purified human and murine enzymes that were active towards 1-menaphthyl sulphate. The absence of a cross-reacting polypeptide in the track which contained rat liver cytosol is probably due to either the low abundance of this GST (see Meyer *et al.*, 1991) or the low titre of the antiserum.

### 11.03 Enzymatic properties of human GST $T_2T_2$

The specific activity of purified human hepatic GST $T_2T_2$ towards a range of substrates is shown in Table 11.01. This enzyme was found to have no detectable activity towards 1-chloro-2,4-dinitrobenzene and, unlike GST $\theta$ described by Meyer *et al.* (1991), it was not active with 1,2-epoxy-3-(*p*-nitrophenoxy)propane. GST $T_2T_2$ was also inactive with the substrate *para*-nitrobenzyl chloride. However, as well as its transferase activity towards 1-menaphthyl sulphate, GST $T_2T_2$ had considerable glutathione peroxidase activity towards cumene hydroperoxide.

### 11.04 Protein sequence analysis of human Theta-class GST

The GST subunit $T_2$ could not be sequenced directly when subjected to automated Edman degradation and was, therefore, concluded to possess a blocked N-terminus. Following digestion of the GST $T_2$ subunit with CNBr, the resulting peptides were resolved by reverse-phase h.p.l.c. (Figure 11.04). Three of the CNBr-derived fragments, represented by protein-containing peaks a, b and c (which eluted at 21, 24 and 28 min, respectively), were collected and subjected to automated amino acid sequencing. The peptide present in peak a was found to possess a blocked N-terminus. Peak b was subjected to 5 cycles

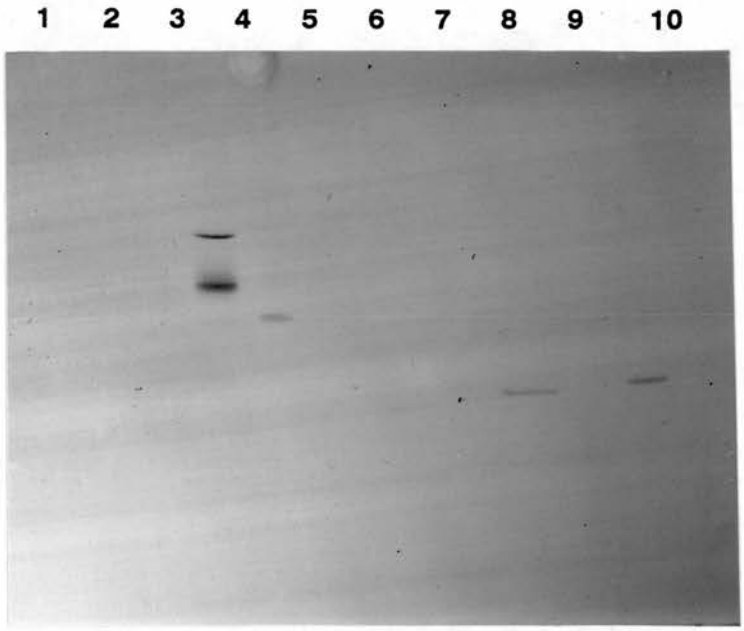



**Figure 11.03 Cross-reactivity of human and mouse Theta-class GST with antisera raised against rat GST E**

Human GST $B_1B_1$, $\mu$, $\pi$, and $T_2T_2$, the mouse GST present in Pool B, rat liver cytosol and samples taken during the purification of GST $T_2T_2$ were probed, following SDS/PAGE and electrophoretic transfer onto nitrocellulose paper, with antisera raised against rat GST E. Protein was applied to the SDS/polyacrylamide gel as follows: tracks 1, 2 and 3, human GST $B_1B_1$, $\mu$ and $\pi$ standards; track 4, rat liver cytosol (30 $\mu$g); track 5, human liver cytosol (30 $\mu$g; specimen L5); track 6, DEAE-cellulose pool (30 $\mu$g); track 7, hydroxyapatite pool (30 $\mu$g); track 8, Matrex™ Gel Orange A pool (3 $\mu$g); track 9, purified GST $T_2T_2$ obtained following Mono Q f.p.l.c. (2 $\mu$g); track 10 mouse chromatofocusing GST Pool B (2 $\mu$g).

**Table 11.01 Specific activities of human GST $T_2T_2$**

For experimental details see Chapter 3. Enzyme assays were performed at 37 °C. Results are expressed as means ± S.D. for three determinations. Abbreviation: N.D., not detected.

| Substrate | Specific activity of GST $T_2T_2$ ($\mu$mol/min per mg) |
|---|---|
| 1-chloro-2,4-dinitrobenzene | N.D. |
| 1-menaphthyl sulphate | 0.497 ± 0.019 |
| 1,2-epoxy-3-(*p*-nitrophenoxy)-propane | N.D. |
| *para*-nitrobenzyl chloride | N.D. |
| Cumene hydroperoxide | 6.9 ± 0.12 |

of Edman degradation, but unfortunately paucity of material would only permit a very tentative identification of the amino acid sequence; the following residues were obtained; leucine, proline, isoleucine, alanine and leucine. The peptide fragment represented by protein peak c, was subjected to 20 cycles of Edman degradation allowing identification of residues 1-21. This sequence is shown in Figure 11.05 aligned for comparison with the homologous N-terminal amino acid sequences of rat YrsYrs (Hiratsuka *et al.*, 1990) as well as rat GST 5-5 and 12-12 and human GST $\theta$ (Meyer *et al.*, 1991). Five amino acid differences are apparent between the N-terminal sequence of the $T_2$ subunit and those of the other Theta-class described in the literature. These changes occur at positions 2, 6, 10, 14 and 17; the amino acid residues are numbered according to the sequence of the $T_2$ subunit. At position 2 the $T_2$ subunit and subunit 5 both contain valine, whereas, subunit 12, $\theta$ and the Yrs subunit all contain glycine. At position 6 the $T_2$ subunit contains a phenylalanine residue, whereas GST 5-5, 12-12 and $\theta$ all contain a tyrosine, and at position 10 GST $T_2$ possesses a valine whilst the other Theta-class GST contain leucine. The amino acid residue at position 14 of the GST $T_2$ subunit has been tentatively identified as serine; GST YrsYrs also contained a serine at this position, whereas, the other rat GST subunits, 5 and 12, both contain cysteine. At position 17 the $T_2$ subunit, as well as GST $\theta$ and subunits 12 and Yrs, contains a valine residue, whereas subunit 5 contains isoleucine.

**Figure 11.04 Preparative peptide map of human GST $T_2T_2$**

A portion (60 $\mu$g) of GST subunit $T_2$, purified by sequential DEAE-cellulose chro
graphy, hydroxyapatite chromatography, Matrex™ Gel Orange A chromatography,
Q f.p.l.c. and reverse-phase h.p.l.c., was digested with CNBr as described in se
3.04a. The resulting CNBr fragments were dissolved in 0.2 ml of aq. 0.1%
trifluoroacetic acid prior to chromatography on an Aquapore RP 300 column (7 $\mu$m p
size; 2.1 mm x 30 mm). The column was developed with a linear 0-70% aceto
gradient in aq. 0.1% (v/v) trifluoroacetic acid, formed over 45 min, as indicated b
straight line. The column eluate was monitored continuously at 220 nm (___).

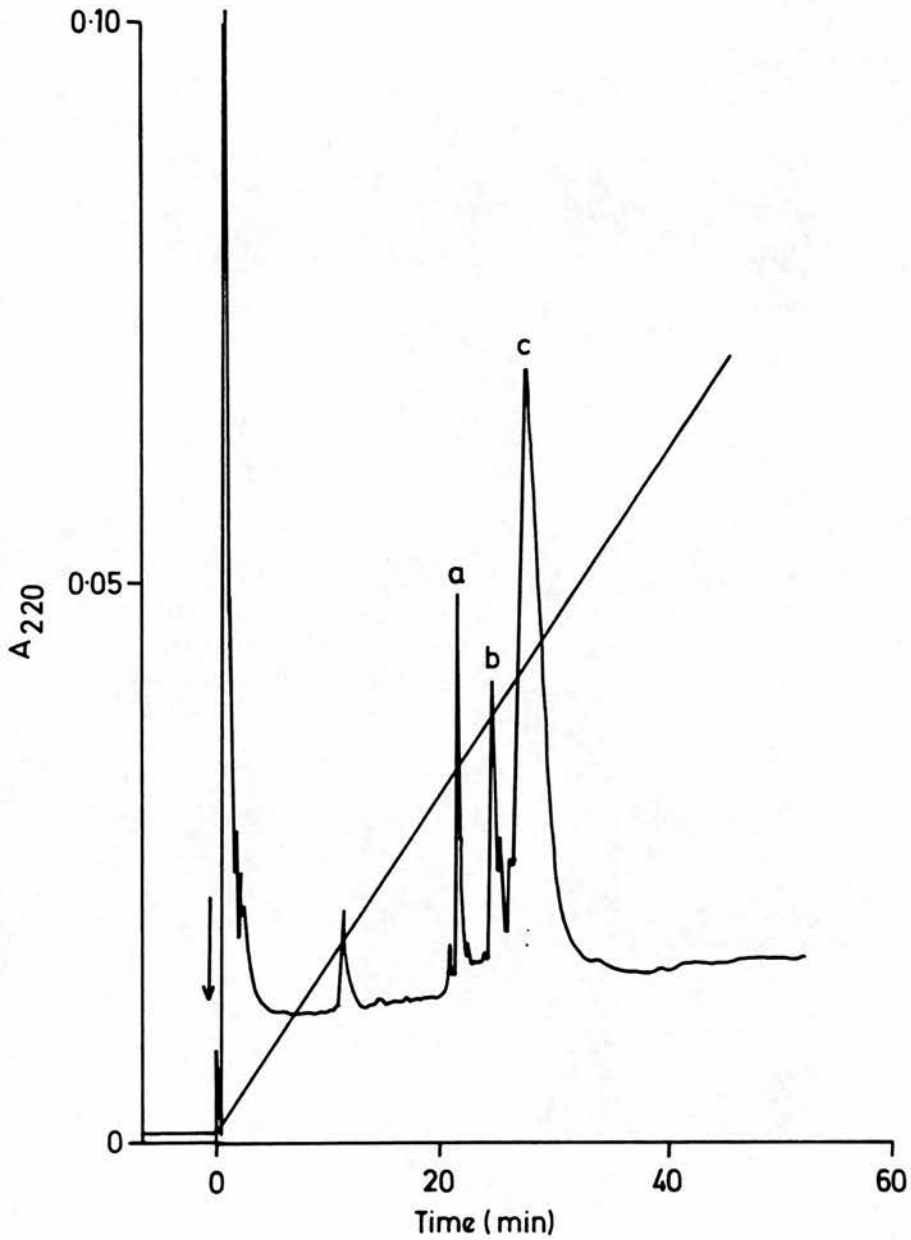



**Figure 11.04**

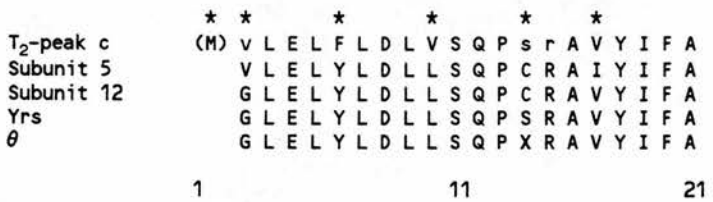



**Figure 11.05 Comparison of the N-terminal amino acid sequence of the $T_2$ subunit with those of other Theta-class GST**

The N-terminal amino acid sequence of subunit $T_2$ was obtained following automated Edman degradation of a CNBr-derived peptide (peak c). This sequence is shown aligned for comparison with the N-terminal amino acid sequences of Theta-class GST 5-5, 12-12, and $\theta$ (Meyer *et al.*, 1991) and GST YrsYrs (Hiratsuka *et al.*, 1990). The putative methionine residue is in parentheses. Where assignments were tentative the residues are shown in lower case. An asterisk (*) indicates amino acid residues that differ between the sequences.

# PART IV

# DISCUSSION

# Chapter 12 Mu-class and Theta-class GST isoenzymes

In this thesis purification schemes have been devised for the isolation of human and murine isoenzymes which belong to the Mu- and Theta-classes of cytosolic GST. The present investigation has shown that the human Mu-class GST are represented by a large number of distinct, but closely related, isoenzymes which are subject to both inter-individual and inter-organ differences in their patterns of expression. The existence of unique GST forms in murine and human liver, which belong to the recently assigned Theta-class, has also been demonstrated.

A number of homodimeric human Mu-class GST are described in the literature which have been named either using the Greek symbols $\mu$, $\psi$ and $\phi$ (Warholm *et al.*, 1983; Singh *et al.*, 1987a; Stockman and Hayes, 1987; Hayes, 1989) or according to their gene loci as GST1 (type 1 and 2), GST4, GST5 and GST6 (Suzuki *et al.*, 1987, 1991; Board *et al.*, 1988). The Mu-class enzymes isolated from human tissues during this project comprise four distinct subunits which may combine to form both homodimeric and heterodimeric proteins. The Mu, or neutral-type subunits, have been designated $N_1^a$, $N_1^b$, $N_2$ and $N_3$, according to the decreasing pI values of the corresponding homodimeric enzymes. This designation permits the use of a more systematic system of nomenclature for the human Mu-class GST which reflects their quaternary structures.

A total of eight separate Mu-class GST have been purified from human tissues during this thesis. Four Mu-class enzymes and one Pi-class enzyme were isolated from skeletal muscle (see Chapter 5). The Mu-class GST are described, in terms of their subunit composition, as $N_1^aN_2$, $N_1^bN_2$, $N_2N_2$ and $N_2N_3$. The $N_1$-type subunits were found in only some of the muscle specimens examined, whereas $N_2$ and $N_3$ were present in all specimens. One homodimeric Mu-class GST, called $N_3N_3$, and an Alpha-class GST were purified from human testicular tissue (see Chapter 6). Several other GST were also present in this specimen, including the Mu-class enzymes $N_1N_2$, $N_2N_2$ and $N_2N_3$, and a Pi-class GST. In addition to these forms, three separate Mu-class isoenzymes, which all comprise $N_1$-type subunits, were isolated from a single human liver specimen (see Chapter 7).

Recently, isoenzymes which belong to a new class of cytosolic GST have been observed in rat and human liver (Hiratsuka *et al.*, 1990; Meyer *et al.*, 1991). During the present investigation a human hepatic Theta-class GST, which catalyses the conjugation of 1-menaphthyl sulphate (1-MS) with GSH, has been purified and is described by its quaternary structure as $T_2T_2$. Theta-class GST forms have also been isolated from mouse liver and shown to have similar properties to those of human GST $T_2T_2$.

A unifying nomenclature for the human GST has recently been proposed by a number of investigators working in this field (Mannervik *et al.*, 1992). This revised nomenclature which provides a designation for each individual subunit, groups the cytosolic enzymes into the four currently recognised classes; Alpha (A), Mu (M), Pi (P) and Theta (T). Arabic numerals may then be assigned to each of the subunits, in the order that they have been described. It is important that isoenzymes are only included formally in the new nomenclature once their entire primary structures are known. Following these guidelines, the Mu-class GST purified during this thesis may be given alternative names using the revised nomenclature of Mannervik *et al.* (1992) (Table 12.01). Although only the N-terminal amino acid sequences of the human Theta-class GST are available at present, these isoenzymes are included in Table 12.01 with an asterix, to indicate a provisional designation.

### 12.01 Mu-class and Theta-class GST present in human liver

The purification of three separate Mu-class GST from one human liver specimen is described in Chapter 7. This specimen contained two homodimeric enzymes, designated $N_1^aN_1^a$ and $N_1^bN_1^b$, and a heterodimeric GST called $N_1^aN_1^b$. Although these isoenzymes possess subunits which have a similar electrophoretic mobility upon SDS/polyacrylamide-gel electrophoresis ($N_1^a$, $M_r$ 27700; $N_1^b$, $M_r$ 27600), they may be distinguished by their isoelectric points. GST $N_1^aN_1^a$ is the most basic of these enzymes, with a pI of 6.1, GST $N_1^bN_1^b$ is the most acidic enzyme, possessing a pI of 5.5, and GST $N_1^aN_1^b$ has an intermediate pI of 5.9. The isoelectric points of the homodimeric GST $N_1^aN_1^a$ and $N_1^bN_1^b$ indicate that these enzymes correspond to GST $\mu$ and GST $\psi$, isoenzymes which have been described previously in the literature (Warholm *et al.*, 1983; Singh *et al.*, 1987a; Hayes, 1989). The purification and characterization of the heterodimeric enzyme, GST $N_1^aN_1^b$, formed by the combination of "$\mu$-type" (or $N_1^a$) and "$\psi$-type" (or $N_1^b$) subunits represents additional information on the human hepatic GST.

**Table 12.01 Revised nomenclature for human Mu- and Theta-class GST**

| Designation used in this thesis | Class | Previous designation[1-7] | 1992 nomenclature[8] |
|---|---|---|---|
| $N_1^aN_1^a$ | Mu | μ, GST1,type 2 | GST M1a-1a |
| $N_1^aN_1^b$ | Mu | N.I. | GST M1a-1b |
| $N_1^bN_1^b$ | Mu | ψ, GST1,type 1 | GST M1b-1b |
| $N_1^aN_2$ | Mu | N.I. | GST M1a-2 |
| $N_1^bN_2$ | Mu | N.I. | GST M1b-2 |
| $N_2N_2$ | Mu | GST4 | GST M2-2 |
| $N_2N_3$ | Mu | N.I. | GST M2-3 |
| $N_3N_3$ | Mu | Brain/Testis μ | GST M3-3 |
| – | Theta | θ | GST T1*-1* or T1*-2* |
| $T_2T_2$ | Theta | N.I. | GST T2*-2* |

References:
1. Warholm *et al.*, 1983
2. Singh *et al.*, 1987
3. Suzuki *et al.*, 1987
4. Board *et al.*, 1988
5. Campbell *et al.*, 1990
6. Vorachek *et al.*, 1991
7. Meyer *et al.*, 1991
8. Mannervik *et al.*, 1992

Abbreviation: N.I., not included

The polymorphism which occurs at the genetic locus encoding the hepatic Mu-class isoenzymes results in the expression of four different phenotypes in human liver. The genetic model proposed by Board (1981) and Strange *et al.* (1984), in order to account for these inter-individual differences, postulated the existence of a heterodimeric protein encoded at this GST1 locus. In a subsequent investigation, which involved chromatofocusing together with hybridization experiments, Faulder *et al.* (1987) demonstrated the presence of such a hybrid enzyme in human liver cytosol. The expected occurrence, in a caucasian population, of this GST1 2-1 phenotype, which results in the expression of at least three Mu-class GST in liver, was estimated by these investigators at approx. 6%.

Recently, Van Ommen *et al.* (1990) quantified the human hepatic GST subunits from a number of individuals, using a reverse-phase h.p.l.c. method. These workers confirmed the genetic polymorphism of subunits $\mu$ and $\psi$, and demonstrated that the combination of both $\mu$ and $\psi$ was present in only one of 20 human liver specimens examined.

The current investigation has shown that GST $N_1^aN_1^a$, $N_1^bN_1^b$, and $N_1^aN_1^b$, the three products of the GST1 locus, possess indistinguishable catalytic properties when examined with a range of compounds (see Table 8.02, Chapter 8). The substrate specificities of the hepatic Mu-class GST isolated during this project are closely similar, with all three isoenzymes containing $N_1$-type subunits having high activity towards *trans*-4-phenyl-3-buten-2-one, *para*-nitrobenzyl chloride and 1-chloro-2,4-dinitrobenzene, as well as moderate activity towards 4-hydroxnonenal, 4-hydroxydecinal and cumene hydroperoxide. The $N_1$-type subunits, however, were found to have no detectable activity towards the compound 1,2-dichloro-4-nitrobenzene. Hayes (1989) purified both GST $\mu$ and GST $\psi$, from two different liver specimens, and also demonstrated that the allelic variants of the human hepatic Mu-class GST are catalytically indistinguishable. The present study confirms several earlier investigations which describe the substrate specificity of GST $\mu$. Others have also observed the high activity of this enzyme towards *trans*-4-phenyl-3-buten-2-one, cumene hydroperoxide and 4-hydroxyalkenals (Warholm *et al.*, 1983; Danielson *et al.*, 1987).

During this thesis it has been shown that, in addition to transferase activity towards CDNB, human liver cytosol contains activity towards the compound 1-menaphthyl sulphate (1-MS). Using GST activity towards this sulphate ester to monitor purification, a homodimeric GST has been isolated from human liver and designated GST $T_2T_2$ (see Chapter 9). This transferase, which has structural homology with the Theta-class GST described by Meyer *et al.*(1991), does not appear to have been purified previously. Other workers have isolated several GST forms from rat liver cytosol which are active towards 1-MS (Gillham, 1973; Hiratsuka *et al.*, 1991) but, to date, the existence of a human GST which can utilize 1-MS as a substrate has not been documented.

Recently, Meyer *et al.* (1991) have isolated a human hepatic Theta-class GST and called it GST $\theta$. A comparison of the physical, catalytic and structural properties of GST $T_2T_2$ with those of GST $\theta$ indicates that these two proteins represent distinct isoenzymes. GST $T_2T_2$ contains two subunits which co-migrate during SDS/polyacrylamide-gel electrophoresis and have a $M_r$ value of 25100; this represents an electrophoretic mobility which is in

between that of the human Alpha-class GST $B_1B_1$ ($M_r$ 26000) and that of the Pi-class GST $\pi$ ($M_r$ 24800). By contrast, electrophoresis of GST $\theta$ gave two bands with mobilities in between those of human Alpha- and Pi-class GST subunits. A comparison of the chromatographic properties of GST $T_2T_2$ with those of GST $\theta$ indicates that these proteins have different charges. GST $T_2T_2$ was retained by Mono Q f.p.l.c. at pH 8.0 (see Chapter 9), whereas, a higher pH (9.55) was necessary to retain GST $\theta$. GST $T_2T_2$ and GST $\theta$ also show significant differences in their substrate specificities. GST $T_2T_2$ not only exhibits activity towards 1-MS, but also has a high glutathione peroxidase activity towards cumene hydroperoxide. Of equal importance is the observation that GST $T_2T_2$ was found to have no detectable activity towards either 1,2-epoxy-*3-(p*-nitrophenoxy)propane or 1-chloro-2,4-dinitrobenzene. By contrast, purified GST $\theta$ was reported to have a low, but detectable, activity towards the epoxide substrate and no detectable activity towards cumene hydroperoxide. Unfortunately, the activity of GST $\theta$ towards 1-menaphthyl sulphate was not documented.

The hypothesis that GST $T_2T_2$ and GST $\theta$ are distinct proteins is supported by the fact that the $T_2$ subunit appeared to possess a blocked N-terminus, whereas, GST $\theta$ is amenable to direct sequence analysis (Meyer *et al.*, 1991). Amino acid sequence analysis of the N-terminal portion of the $T_2$ subunit was achieved by automated Edman degradation following CNBr cleavage of the protein. A plausible reason why the $T_2$ subunit has a blocked N-terminus is that the initiator methionine amino acid remains attached to the protein and is acetylated by a post-translational event. It is of interest that the chemical nature of the blocked group at the N-terminus of rat GST subunit 8 (or Yk) has recently been identified as an acetylmethionine structure (Alin *et al.* 1989). The amino acid sequence of the N-terminal region of the GST $T_2$ subunit was found to be closely similar to the published N-termini of other Theta-class GST (Hiratsuka *et al.*, 1990; Meyer *et al.*, 1991); no apparent homology was noted between $T_2$ and other GST isoenzymes which belong to classes Alpha, Mu or Pi. Although the $T_2$ subunit and GST $\theta$ have similar N-terminal amino acid sequences, three conservative amino acid substitutions are present in the first 20 residues (see Figure 11.05). At positions 2, 6 and 10 the $T_1$ subunit contains valine, phenylalanine and valine, whereas the GST $\theta$ subunit contains glycine, tyrosine and leucine, respectively.

The immunochemical data presented in this thesis supports the assignment of GST $T_2T_2$ to the Theta-class; no immunological cross-reactivity was observed between GST $T_2T_2$ and antisera raised against human Alpha-, Mu- and Pi-class GST. By contrast, the GST $T_2$

subunit was found to be related immunochemically to antisera raised against the original GST E preparation obtained from rat liver described by Fjellstedt *et al.* (1973).

The precise relationship between the homodimeric Theta-class GST isolated during this project and the hepatic enzyme identified by Meyer *et al.* (1991) is unclear. Whilst the data presented in this thesis indicate that GST $T_2T_2$ and GST $\theta$ represent separate isoenzymes, the electrophoretic data reported by Meyer *et al.* (1991) indicate that GST $\theta$ may be a heterodimeric protein. Therefore the possibility that the two enzymes share a common subunit cannot be excluded.

## 12.02 Extra-hepatic expression of human GST isoenzymes

This thesis describes the purification of a total of five separate Mu-class GST from human extra-hepatic tissues. The skeletal muscle and testis forms isolated during this investigation comprise both homo- and heterodimeric proteins formed by the combination of four different N-type subunits. Previously, several other groups of workers have identified a number of extra-hepatic human GST (Laisney *et al.*, 1984; Suzuki *et al.*, 1987; Board *et al.*, 1988; Singh *et al.*, 1988; Campbell *et al.*, 1990; Singhal *et al.*, 1991). The relationships between the transferases isolated during this project and those described elsewhere will be discussed.

### 12.02a Characterization of the N-type GST subunits

The skeletal muscle GST $N_1{}^bN_2$, $N_2N_2$, $N_2N_3$ and a homodimeric GST $N_3N_3$ isolated from a human testis sample possess distinct catalytic properties. The substrate specificities of these isoenzymes are also clearly distinct from those of the hepatic Mu-class GST (see Table 8.01). The $N_1{}^bN_2$ heterodimer, together with the hepatic Mu-class isoenzymes ($N_1{}^aN_1{}^a$, $N_1{}^bN_1{}^b$, and $N_1{}^aN_1{}^b$) all possess a high activity towards *trans*-4-phenyl-3-buten-2-one and *para*-nitrobenzyl chloride. This is an indication that the $N_1$-type subunits are responsible for catalysing the conjugation of GSH to these compounds. All of the Mu-class GST purified from skeletal muscle were active towards 1,2-dichloro-4-nitrobenzene. By contrast, the hepatic isoenzymes were inactive towards this compound. The $N_2$ subunit is, therefore, strongly implicated in the metabolism of 1,2-dichloro-4-nitrobenzene. The $N_1$-type, $N_2$ and $N_3$ subunits were all found to possess a high transferase activity towards 4-hydroxynonenal and 4-hydroxydecinal. Other workers have also noted the high activity of the hepatic enzyme GST $\mu$ towards 4-hydroxyalkenals (Danielson *et al.*, 1987).

Unfortunately, no substrate was identified that could serve as a marker for the $N_3$ subunit. However, the testis enzyme GST $N_3N_3$ was distinguished by a significantly lower activity towards 1-chloro-2,4-dinitrobenzene than that observed for other human Mu-class GST.

With the exception of GST $N_2N_3$ and $N_3N_3$, the human Mu-class GST purified during this investigation have different isoelectric points (see Figure 8.01 and Table 8.01). However, the pI values of these enzymes fall within a close range (between 6.1 and 5.0), therefore, it is unwise to identify the GST solely on the basis of their isoelectric points. The subunit types that the Mu-class GST comprise can be resolved conveniently and identified by SDS/PAGE. With this method the $N_1$-type, $N_2$ and $N_3$ subunits have estimated $M_r$ values of 26700, 26000 and 26300 respectively. It is important to recognise that the $N_2$ subunit and the Alpha-class $B_1/B_2$ subunits co-migrate during SDS/PAGE and that immunochemical methods are required to help discriminate between these subunits.

Subunits $N_1^a$, $N_1^b$, $N_2$ and $N_3$ showed no cross-reactivity towards antibodies raised against either human Alpha-class GST $B_1B_1$ or the Pi-class GST $\pi$. All of the N-type subunits were found to cross-react with antisera raised against hepatic GST $\mu$ (or $N_1^aN_1^a$) and, therefore, although the $N_2$ and $B_1/B_2$ subunits co-migrate during SDS/PAGE, they can be discriminated immunochemically. The data also suggest that the individual N-type subunits can be distinguished by immunochemistry, but that to achieve identification the choice of antiserum is important (Table 12.02).

**Table 12.02 Immunochemical properties of human N-type subunits**

| Subunit | Cross-reactivity with antisera against human GST: | | | | |
|---|---|---|---|---|---|
| | $B_1B_1$ | $\pi$ | $N_1^aN_1^a$ | $N_2N_2$ | $N_3N_3$ |
| $N_1^a$ | - | - | ++++ | ++++ | - |
| $N_1^b$ | - | - | ++++ | ++++ | - |
| $N_2$ | - | - | ++ | ++++ | - |
| $N_3$ | - | - | + | - | ++++ |

The degree of cross-reactivity towards antibodies against GST μ (or $N_1^aN_1^a$) was found to vary from subunit to subunit. As expected, the $N_1$-type subunits cross-reacted strongly with this antiserum, whereas the $N_2$ and the $N_3$ subunits both showed a weaker reactivity. Antibodies raised against the $N_2$ subunit were found to cross-react with the $N_1$ subunit, but not with the $N_3$ subunit. By contrast, anti-($N_3N_3$) antibodies only cross-reacted with the $N_3$ subunit and, therefore, clearly distinguish this subunit from other human Mu-class GST.

N-terminal amino acid sequencing suggests that the $N_1$-type, $N_2$ and $N_3$ subunits are genetically separate (see Figure 8.05). It was possible to analyze the purified $N_2$ and $N_3$ subunits directly. However, the constituent subunits of GST $N_1^aN_2$ were not resolved by reverse-phase h.p.l.c. and, therefore, the sequence data presented for subunit $N_1^a$ were derived from the analysis of the $N_1^aN_2$ heterodimer. Comparison between $N_1^a$ and $N_2$ showed differences at residues 3, 8 and 15; at these positions subunit $N_1^a$ appeared to contain isoleucine, aspartic acid and alanine, whereas subunit $N_2$ was found to contain threonine, asparagine and serine. Comparison of the N-terminal sequence data for skeletal muscle subunit $N_1^a$ with the primary structure of the subunits of hepatic GST μ suggest that they are identical. This conclusion is supported by the immunochemical results, the observation that the $N_1$-type subunit has a high activity for *trans*-4-phenyl-3-buten-2-one and the inter-individual variability in the expression of the $N_1$-type subunits. In contrast to the $N_1^a$ and the $N_2$ subunits, the $N_3$ subunit was found to possess a blocked N-terminus. Recently, Campbell *et al.* (1990) have purified a Mu-class GST from human testis, which they have called brain/testis μ. It is of interest to note that this isoenzyme was reported to have a pI of 5.2 which is similar to that of GST $N_3N_3$ purified during this study, and to possess a blocked N-terminus.

Inspection of the sequence data for the $N_2$ subunit compared with the cDNA clone encoding GST μ (DeJong *et al.*, 1988) shows that they are genetically distinct (see Figure 8.06); differences in primary structure were noted at residues 3, 8, 15, 66, 77, 133, 139, 144, 148 and 150. Moreover, comparison of these data for the $N_2$ subunit with the N-terminal sequence for the skeletal muscle GST4 described by Board *et al.* (1988) suggests that subunits $N_2$ and GST4 are probably the same. The amino acid sequence analysis of subunit $N_2$ also allowed comparison with the genomic human Mu-class GST clones of Taylor *et al.* (1990, 1991). This indicated that $N_2$ probably represents the subunit encoded by clone GST mu3. Recently, Vorachek *et al.* (1991) have isolated a cDNA clone (called GTHMUS) encoding a GST expressed in human myoblasts. Protein sequence data obtained by these workers indicates that this skeletal muscle GST is equivalent to the

protein encoded by the GST4 locus described by Board *et al.* (1988). A comparison of the amino acid sequence of the $N_2$ subunit with the deduced amino acid sequence of the human muscle cDNA clone GTHMUS indicates that they are identical, with the exception of one amino acid residue. At position 209 the $N_2$ subunit contains a serine residue, whereas the protein encoded by GTHMUS contains threonine. It is possible that this single conservative amino acid substitution is a result of allelic variation.

The $N_3$ GST subunit, which is expressed in both skeletal muscle and testis tissue, was found to possess a blocked N-terminus. A preparative CNBr digest of the $N_3$ subunit purified from human testicular tissue, however, enabled a small region of the protein, between residues 188-208, to be sequenced. The 14 amino acids identified in this region were found to be identical to the corresponding amino acids encoded by a human cDNA clone HTGT-6 isolated from a human testis library (Campbell *et al.*, 1990). These data are further confirmation of the identity of GST $N_3N_3$ with the brain/testis $\mu$ isoenzyme described by Campbell *et al.* (1990).

Others have studied the GST isoenzyme content of human skeletal muscle. Awasthi and co-workers isolated a number of GST from muscle which have N-terminal sequences either identical to or closely related those of the $N_1^a$ or the $N_2$ subunit (Singh *et al.*, 1988; Singhal *et al.*, 1991). The major muscle isoenzyme which was described by these workers, however, had a blocked N-terminus. This transferase, called GST $\zeta$, had an acidic pI (5.2) and did not cross-react with antibodies raised against either human Alpha-, Mu- or Pi-class GST. Although the $N_3$ subunit is not an abundant protein in the muscle specimens analysed during this project, it is a possibility, given the similarities in structural and immunochemical properties between the two proteins, that GST $N_3N_3$ is related to GST $\zeta$ observed by Singhal *et al.* (1991). It is also worth noting that the Mu-class homodimer GST $\phi$, which Stockman and Hayes (1987) found in only one of 20 human liver specimens, may also represent GST $N_3N_3$ as it also possessed a blocked N-terminus.

12.02b Variation in the expression of $N_1$-type GST subunits in extra-hepatic tissues

Each of four skeletal muscle specimens (M1, M2, M3 and M4; see Table 4.01) included in this study had a different Mu-class GST isoenzyme content. These differences were due to a variation in the expression of the $N_1$-type subunits. All of the muscle specimens examined contained GST $N_2N_2$ and GST $N_2N_3$. However, specimens M2, M3 and M4 expressed additional heterodimeric enzymes. Muscle specimen M2 contained GST $N_1^aN_2$,

specimen M3 contained GST $N_1^bN_2$ (see Chapter 5), and muscle specimen M4 expressed both of these GST forms (see Chapter 7).

In order to establish the relationship between the expression of $N_1$-type GST subunits in human liver and the presence of these subunits in extra-hepatic tissues, the isoenzyme profiles from several different tissue samples obtained from the same individual were analysed (see Chapter 7). A liver specimen obtained from this individual expressed subunits $N_1^a$ and $N_1^b$, two of the allelic variants encoded by the hepatic Mu-class GST locus. Analysis of the GST content of skeletal muscle and cerebrum indicated that the extra-hepatic tissues both expressed two chromatographically distinct heterodimeric proteins which contained an $N_1$-type subunit together with an $N_2$ subunit. Therefore, the hepatic Mu-class GST phenotype of this individual influenced the Mu-class GST subunits expressed in skeletal muscle and cerebrum. Others have observed a direct relationship between the expression of GST $\mu$ in liver its presence in mononuclear leukocytes (Seidegard *et al.*, 1987).

The work presented in this thesis clearly demonstrates the ability of the skeletal muscle $N_2$ subunit to hybridize with other Mu-class subunits. The existence of a heterodimeric protein formed by the combination of the subunits encoded by the GST1 locus (i.e. $N_1^a$ and $N_1^b$) and the GST4 locus (i.e. $N_2$) was first predicted, by using zymogram analysis, by Laisney *et al.* (1984). This finding, however, was not confirmed when similar studies were performed by Suzuki *et al.* (1987). The isolation and characterization, described in this thesis, of the $N_1^aN_2$ and $N_1^bN_2$ heterodimeric GST, therefore, supports the earlier work of Laisney *et al.* (1984). The purification of the $N_2N_3$ heterodimer from skeletal muscle represents new information on the multiplicity of the human Mu-class GST family. During the present study a $N_1N_3$ heterodimer was not recovered from either skeletal muscle or testis and although its existence seems probable there is no obvious explanation for its absence. Campbell *et al.* (1990) have also noted the absence of a natural heterodimer formed by the combination of Mu-class GST subunits obtained from human liver and testis.

### 12.03 The existence of multiple human Mu-class GST

The purification and characterization, described in this thesis, of three genetically distinct N-type subunits supports the view that the human Mu-class GST are members of a multigene family. Others have also described extra-hepatic Mu-class isoenzymes which

appear to exhibit a degree of tissue-specific expression. A brain form, termed GST5, has been identified and purified and shown to be related to the GST1 (or $N_1$-type) isoenzymes (Laisney *et al.*, 1984; Suzuki *et al.*, 1987). This GST was reported to have a pI of 5.9, which falls between $N_1^aN_1^a$ and $N_1^bN_1^b$, and an identical subunit $M_r$ to that of the product of the GST1 locus. In addition, this GST cross-reacted readily with antisera raised against GST1 isoenzymes. An acidic GST with a pI of 4.25, called GST6, has also been observed in human tissues (Suzuki *et al.*, 1987). The purification of this form from human brain and its subsequent characterization has revealed that it has an identical N-terminal amino acid sequence to that of GST4 (Board *et al.*, 1988; Suzuki *et al.*, 1991). GST6 cross-reacted with antisera raised against GST4, but not with antisera raised against either GST1, GST2 or GST3. Furthermore, this GST has an electrophoretic mobility on SDS/PAGE that is distinct from those of GST1 and GST4.

Recently, Tsuchida *et al.* (1990), have isolated five Mu-class subunits from human heart and aorta. These subunits were shown to form a combination of homo- and heterodimeric proteins. The three isoenzymes isolated from human heart, by these workers, could be identified as GST $N_1^aN_1^a$, $N_1^aN_2$ and $N_2N_2$ on the basis of their isoelectric points and N-terminal amino acid sequences. However, two of the three subunits which comprise the two proteins purified from aorta had distinct amino acid substitutions within their amino terminal sequence. Therefore, in addition to the N-type subunits described in the present study, there is evidence in the literature for the existence of multiple Mu-class GST which are subject to tissue specific expression and may be the products of additional Mu-class genes.

A total of four full-length cDNA clones which encode four separate Mu-class GST subunits have been isolated by different workers. The Mu-class isoenzyme forms which these cDNA represent have all been purified and characterised. Two of these cDNA encode the hepatic isoenzymes GST $\mu$ (or $N_1^aN_1^a$) and GST $\psi$ (or $N_1^bN_1^b$) which differ by a single amino acid at position 172 (DeJong *et al.*, 1988; Seidegard *et al.*, 1988; Widersten *et al.*, 1991). A third human Mu-class cDNA has a mostly divergent 3′ non-coding region and encodes a protein expressed in testicular and brain tissue. The GST encoded by this cDNA clone (called testis/brain $\mu$) shares approx. 68% protein sequence homology with the hepatic enzymes (Campbell *et al.*, 1990). Recently, Vorachek *et al.* (1991) have isolated a fourth cDNA clone which encodes a skeletal muscle Mu-class enzyme. This cDNA clone has a high homology in the 3′ non-coding region with that of the hepatic cDNA clones identified earlier. In addition, the protein encoded by the skeletal muscle

cDNA clone shares approx. 85% homology with the GST1 isoenzymes. A comparison of the N-terminal amino acid sequence of the protein encoded by the muscle cDNA with that of GST4, described by Board *et al.* (1988), indicates that these proteins may be identical. This view is further supported by a comparison of the physical properties of the purified enzymes.

A recent study has indicated that the human Mu-class GST may be encoded by a dispersed gene family (DeJong *et al.*, 1991). The genomic organization of four human Mu-class genes, with related 3′ non-coding sequences, was investigated by these workers using a panel of mouse-human somatic cell hybrids. The hybridization analysis revealed that the four DNA fragments segregated with three different human chromosomes. An 8 kb EcoR1 fragment, which appeared to represent the polymorphic hepatic Mu-class subunit genes, segregated with chromosome 13. Two fragments of 5.1 kb and 4.3 kb co-segregated with chromosome 1, consistent with earlier *in situ* hybridization results obtained using a GST μ cDNA probe (DeJong *et al.*, 1988). A further 10 kb DNA fragment segregated with chromosome 6. In this context it is worth noting that a cosmid clone analysis by Taylor *et al.* (1991) indicated that at least two of the Mu-class genes (called mu2 and mu3) are closely linked.

The evolutionary origin of the multiple human Mu-class GST has been the subject of speculation in the literature. A number of workers have proposed that gene conversion events are responsible for the high degree of homology seen in the coding and non-coding regions of some of the human Mu-class genes (Taylor *et al.*, 1991; Vorachek *et al.*, 1991). A more recent proposal, which may account for the observation that the human Mu-class gene family is dispersed, is that the polymorphic GST μ/ψ gene was created by a transposition or recombination event during evolution (DeJong *et al.*, 1991).

### 12.04 Inter-species relationships between Mu-class and Theta-class GST

The multiplicity observed in the human cytosolic GST is also apparent in other mammalian species. For example, both rat and murine Mu-class GST are represented by multiple isoenzymes which, although related, are catalytically and structurally distinct (Hayes, 1984; Tu and Reddy, 1985; Ishikawa *et al.*, 1988; Hayes *et al.*, 1991b). To date, only three rat Theta-class GST have been described (Hiratsuka *et al.*, 1990; Meyer *et al.*, 1991; Harris *et al.*, 1991), therefore, information on the complexity of a possible fourth GST multigene family has yet to be obtained.

12.04a A comparison of human and rat Mu-class GST isoenzymes

In the rat multiple Mu-class Yb-type and Yn-type GST subunits have been identified (Hayes, 1984; Ishikawa *et al.*, 1988). The dimeric enzymes which are composed of these subunits, together with an additional subunit type, named Yo (Hayes, 1988; Kispert *et al.*,1989), are subject to a marked tissue specific expression. Rat liver cytosol contains at least two Yb-type subunits (called $Yb_1$ and $Yb_2$) which can combine to form a homodimeric and two heterodimeric proteins (Hayes, 1983; Tu and Reddy, 1985). Furthermore, the presence in rat liver of a Yn-type subunit which may form heterodimeric proteins with both the $Yb_1$ and the $Yb_2$ subunits has also been demonstrated (Hayes, 1984). More recently, Ishikawa *et al.* (1988) have shown that rat testis contains two distinct Yn-type subunits. The major GST form isolated from this tissue was a heterodimeric protein comprising $Yn_1$ and $Yn_2$ subunits. A $Yn_1Yn_1$ homodimer was not purified from rat testis by these workers, however, it was found in rat brain. These investigators also demonstrated that subunits $Yn_1$ and $Yb_2$ can combine to form a heterodimer which is detectable not only in rat testis but also in heart kidney and lung. Others have described an additional Mu-class GST, called YoYo (or 11-11), which is also expressed in rat testis (Hayes, 1988; Kispert *et al.*, 1989).

A variation in the GST isoenzyme content of different human tissues was also clearly evident during the present study. The GST subunits $N_2$ and $N_3$ were found to be absent from human liver, however, both of these proteins were expressed in skeletal muscle and testis. The $N_2$ subunit was a major form in skeletal muscle, as were the heterodimeric proteins formed by the combination of the $N_2$ subunit with either $N_1$-type, when present, or $N_3$ subunits. In addition to GST $N_1N_2$ and $N_2N_2$, the homodimeric GST $N_3N_3$ was found to be a major form present in the human testis sample analysed. The $N_3$ subunit is distinct from the $N_1$-type and $N_2$ subunits in that it possesses a blocked N-terminus. This is an interesting observation, as in the rat the only Mu-class GST subunit that has a blocked N-terminus is Yo (Hayes, 1988; Kispert *et al.*, 1989), the $Yb_1$, $Yb_2$, $Yn_1$ and $Yn_2$ subunits having N-termini that are amenable to direct sequencing analysis. It is, therefore, possible that the human subunit $N_3$ is the homologue of the rat Yo subunit.

It is worth noting the relationships between the substrate specificities of the human N-type subunits and those of rat Mu-class isoenzymes (Table 12.03). Each of the rat GST subunits has characteristic specific activities for certain substrates. The compounds *trans*-4-phenyl-3-buten-2-one (*t*-PBO), 1,2-dichloro-4-nitrobenzene (DCNB), bromosulpho-

phthalein (BSP) and *para*-nitrobenzyl chloride (*p*-NBC) may be used to distinguish between the rat $Yb_1$, $Yb_2$ and $Yn_1$ subunits (Mannervik and Jenssen, 1982; Hayes, 1986). For example, the rat GST $Yb_2Yb_2$ has a high specific activity for *t*-PBO and a relatively low activity for DCNB, whereas the converse is true for both rat GST $Yb_1Yb_1$ and $Yn_1Yn_1$. The latter enzymes may be distinguished by their respective activities towards the compounds *p*-NBC and BSP. These four substrates may also be used to discriminate between the human N-type subunits. If one examines the catalytic activities of the human isoenzymes it is apparent that GST $N_2N_2$ has a similar substrate specificity to that of the rat enzyme $Yn_1Yn_1$, whereas the substrate preferences of the homodimeric enzymes containing $N_1$-type subunits are more closely similar to that of rat GST $Yb_2Yb_2$. The catalytic activities of the human and rat Mu-class isoenzymes towards 1-chloro-2,4-dinitrobenzene (CDNB) are also of interest. Human GST $N_2N_2$ and rat GST $Yn_1Yn_1$ both have a characteristically high activity towards this substrate. By contrast, CDNB was found to be a relatively poor substrate for both human GST $N_3N_3$ and rat GST YoYo.

Other investigators have also noted similarities between the substrate preferences of certain human Mu-class GST and those of rat Mu-class GST. Tsuchida *et al.* (1990) have purified three isoenzymes from human heart which appear to be identical to the GST $N_1^aN_1^a$, $N_1^aN_2$ and $N_2N_2$ described in this thesis. These workers concluded, using the activities of the different enzymes towards DCNB to distinguish between GST forms, that GST $N_2N_2$ is equivalent to rat GST $Yn_1Yn_1$, and proposed that the human enzyme GST $N_1^aN_2$ may correspond to GST $Yb_2Yn_1$ which others have detected in rat heart and testis (Ishikawa *et al.*, 1988). Vorachek *et al.* (1991) found that activity towards *t*-PBO may be used to discriminate between the muscle enzyme GST4 (or $N_2N_2$) and the hepatic GST $\psi$ (or $N_1^bN_1^b$). This investigation demonstrated that human GST $\psi$ and rat GST $Yb_2Yb_2$ share a high specific activity towards *t*-PBO, whereas human GST4 and rat $Yn_1Yn_1$ have a low activity towards this compound. Following a comparison of the amino acid sequences of GST $\psi$ and GST4 with those of the rat $Yb_2$ and $Yn_1$ subunits, these workers have identified a region between residues 158-168 which, they believe, may be involved in the catalysis of *t*-PBO. In this region the amino acid sequence of $Yb_2$ is identical to that of GST $\psi$, whereas that of GST4 differs from the rat $Yb_2$ subunit at 7 of the 11 residues.

Western blot analysis has revealed that the $N_1$-type, $N_2$ and $N_3$ subunits are immunochemically related to a number of rat Mu-class GST, however, the degree of cross-reactivity observed was dependant on the particular antisera used in each case (see

**Table 12.03 Catalytic properties of human and rat Mu-class GST**

| Substrate | Specific activity ($\mu$mol/min per mg) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Human GST | | | Rat GST[1-3] | | | |
| | $N_1{}^aN_1{}^a$ | $N_2N_2$ | $N_3N_3$ | $Yb_1Yb_1$ | $Yb_2Yb_2$ | $Yn_1Yn_1$ | YoYo |
| CDNB | 190 | 277 | 15.2 | 186 | 34 | 248 | 30 |
| *t*-PBO | 0.2 | N.D. | N.D. | 0.2 | 3.0 | 0.1 | - |
| DCNB | N.D. | 2.0 | N.D. | 10.7 | 0.1 | 2.3 | 0.33 |
| BSP | N.D. | N.D. | N.D. | 1.6 | N.D. | N.D. | - |
| *p*-NBC | 2.7 | N.D. | N.D. | 14.0 | 14.4 | 1.7 | 0.5 |
| 4-HNE | 3.3 | 3.6 | 1.8 | 2.7 | 6.9 | - | 2.1 |



References:
1. Hayes *et al.* (1986)
2. Danielson *et al.* (1987)
3. Kispert *et al.* (1989)

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; *t*-PBO,*trans*-4-phenyl-3-buten-2-one; DCNB,1,2-dichloro-4-nitrobenzene; BSP, bromosulphophthalein; *p*-NBC, *para*-nitrobenzyl chloride; 4-HNE, 4-hydroxynonenal; N.D., not detected.

Figure 8.04; Chapter 8). The $N_1$-type subunits cross-reacted strongly with antisera raised against rat GST $Yb_1Yb_1$, $Yn_1Yn_1$ and YoYo, whereas a strong cross-reactivity with the $N_3$ subunit was only evident using antisera raised against either rat GST $Yb_1Yb_1$ or GST YoYo. By contrast, a weak cross-reactivity was observed between the $N_2$ subunit and all three rat Mu-class GST antisera. Tsuchida *et al.* (1990) have examined the relationships between human and rat Mu-class GST using immunodiffusion. These workers have reported that immunochemical relationships exist between the human GST which corresponds to $N_1^aN_1^a$ and rat GST $Yb_2Yb_2$, and between the human enzyme which represents GST $N_2N_2$ and rat $Yn_1Yn_1$.

The analysis of genomic DNA using Southern blotting experiments has indicated that a multigene family encode the rat Mu-class GST (Lai *et al.*, 1986). In agreement with this observation a number of cDNA clones which encode separate Mu-class GST subunits expressed in rat liver (Ding *et al.*, 1986) and testis (Abramowitz and Listowsky, 1987) have been isolated. In addition, the structural genes which encode three rat liver Yb-type subunits have been isolated and characterized (Lai *et al.*, 1988; Morton *et al.*, 1990). Two of these genes encode the rat $Yb_1$ and $Yb_2$ subunits, however, the third gene encodes a subunit, designated $Yb_4$, which has not been purified to date from any rat tissue. A comparison of the nucleotide sequences of genes encoding the $Yb_1$ and $Yb_2$ subunits reveals that all of the exons and some of the introns share significant identity (Morton *et al.*, 1990). Tu and co-workers have also reported a conservation of the introns in the genes encoding subunits $Yb_1$ and $Yb_4$ (Lai *et al.*, 1988). Therefore, as with the human Mu-class genes, there is evidence for gene conversion in the evolution of the rat Mu-class multigene family. The recent analysis of the human genomic Mu-class genes by Taylor *et al.* (1991) has indicated that the exon-intron boundaries are conserved between man and rat. This study also demonstrated that there is a high degree of nucleotide sequence homology in both the exons and some of the introns between sequences of the human mu2 and mu3 genes and the rat $Yb_2$ subunit gene. These workers proposed that the conservation of introns between human and rat Mu-class genes was possibly due to the presence of regulatory sequences.

12.04b Theta-class GST present in human, murine and rat liver

An investigation into hepatic GST which catalyse the conjugation of GSH to 1-menaphthyl sulphate has been undertaken during this thesis. This has led to the isolation and characterization of a homodimeric human isoenzyme and the identification of related murine GST forms.

The earliest reports on the chromatographic purification of GST employed either 1-menaphthyl sulphate (1-MS) or 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) as substrates for these enzymes (Gillham, 1971,1973; Fjellstedt *et al.*, 1973). With the development of a general purification scheme it was found that most of the rat liver GST catalyse the conjugation of GSH to 1-chloro-2,4-dinitrobenzene (CDNB) and, therefore, this compound was adopted as the substrate of choice to monitor purification (Habig *et al.*, 1974a, 1976). The widespread, almost exclusive use of CDNB as a GST substrate, however, has resulted in the GST which metabolise 1-MS and EPNP being ignored until relatively recently. In rat liver cytosol separate GST isoenzymes are responsible for metabolising 1-MS and EPNP. GST YrsYrs, described by Hiratsuka *et al.* (1990), was shown to be active with 1-MS but not EPNP. Three Theta-class GST (referred to as 5-5, 12-12 and 13-13) have been isolated by Ketterer and co-workers, and it appears that only one of these transferases, possibly 12-12, is active with 1-MS (Harris *et al.*, 1991; Meyer *et al.*, 1991).

The current investigation has indicated that human liver also contains separate GST which are responsible for the metabolism of 1-MS and EPNP. GST $T_2T_2$, purified during this study, catalyses the conjugation of GSH to 1-MS. However, unlike the human GST $\theta$ described by Meyer *et al.* (1991), this isoenzyme is not active towards the epoxide substrate EPNP. This study has also shown that male mouse liver contains GST forms that metabolise 1-MS. Unfortunately, due to paucity of material, the activity of a purified murine enzyme towards EPNP has not been established. However, it is worth noting that murine fraction obtained following hydroxyapatite chromatography that was active with 1-MS did not possess EPNP activity. In addition to the GST activity towards 1-MS, the human GST $T_2T_2$ exhibited a high peroxidase activity towards cumene hydroperoxide. Interestingly, peroxidase activity, with cumene hydroperoxide as a substrate, was also attributed to rat GST YrsYrs (Hiratsuka *et al.*, 1990) and rat GST 5-5 (Meyer *et al.*, 1991). Comparisons between the physical properties of the human, murine and rat GST which metabolise 1-MS reveals a number of similarities. The GST $T_2$ subunit present in the human enzyme had a similar, but not identical, electrophoretic mobility on SDS/PAGE to

that of the subunit present in the murine 1-MS active GST. GST $T_2T_2$ has a subunit $M_r$ of 25100, whereas the mouse enzyme has a subunit $M_r$ of 25400. The murine GST forms have pI values, which may be estimated from chromatofocusing, in the region of 8.3-8.0. It is noteworthy that GST YrsYrs described by Hiratsuka *et al.* (1990) also eluted from chromatofocusing columns between pH 8.2-8.0.

The subunits present in the human GST $T_2T_2$ and the mouse 1-MS metabolising GST are both immunochemically related to a preparation of rat liver GST E, which catalyses the conjugation of GSH to EPNP. In addition, the human enzyme shares N-terminal amino acid sequence homology with the rat liver GST which metabolise either 1-MS or EPNP. Therefore, the immunochemical and the sequence data are a clear indication that the human and the murine isoenzymes, described in this study are novel members of the Theta-class of GST. The entire coding sequence of the rat Yrs subunit was revealed recently following the isolation of a rat liver cDNA (Ogura *et al.*, 1991). This cDNA encoded a polypeptide which contained 244 amino acid residues, the sequence of which showed very little homology (19-23%) with those of rat liver isoenzymes belonging to classes Alpha, Mu and Pi.

Recently, a rat liver mitochondrial matrix GST has been purified and shown to have a structural similarity to the rat cytosolic Theta-class enzymes (Harris *et al.*, 1991). A comparison of the N-terminal amino acid sequence of mitochondrial GST 13-13 with that of human GST $T_2T_2$ reveals that 8 of the first 15 residues present in the $T_2$ subunit are identical to those found in subunit 13. One of these residues, namely phenylalanine at position 6, is not found in the corresponding position in any of the other known cytosolic Theta-class GST.

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subunit $T_2$ | | | | | (M)v | L | E | L | F | L | D | L | V | S | Q | P | s | r |
| Subunit 13 | C | P | A | P | R | V | L | E | L | F | Y | D | V | L | S | P | Y | S | X |
| | | | | | 1 | | | | | | | | | | | | | 15 | |

Harris *et al.* (1991) have also noted structural similarities between GST 13-13 and the dichloromethane dehalogenase of *Methylobacterium sp.* (La Roche and Leisinger, 1990). Therefore, it is possible that an evolutionary relationship exists between the bacterial, mitochondrial and Theta-class cytosolic GST of eukaryotes.

## 12.05 Biological consequences of variation in GST isoenzyme expression

The GST isoenzymes clearly play an important role in cellular defence mechanisms which exist to combat the cytotoxic and genotoxic effects of both endogenous and xenobiotic electrophiles. Although these enzymes display a broad and overlapping range of substrate specificities, each of the separate classes of GST show a characteristic substrate preference. For example, Mu-class GST are efficient in the detoxification of mutagens such as styrene oxide and *trans*-stilbene oxide, and carcinogenic epoxides such as benzo(a)pyrene-7,8-diol-9,10-epoxide (Seidegard *et al.*, 1987; Ketterer, 1988, Mannervik and Danielson, 1988). Whereas, Theta-class GST have been shown to catalyse the conjugation of GSH to a number of highly reactive sulphate esters formed from the corresponding hydroxymethylarenes (Hiratsuka *et al.*, 1990).

The work presented in this thesis has shown that within a class the individual subunits, which comprise the human enzymes, display distinctive activities towards certain substrates. The GST isoenzyme pattern present in a particular tissue may, therefore, have both toxicological and pharmacological implications. In man, the GST are subject to both inter-individual and tissue specific expression. The variations observed in isoenzyme profiles are thought to be influenced by a number of factors; these include genetic deficiencies, developmental changes, hormonal influences, as well as the sensitivity of the individual GST forms towards inhibitors and inducers.

The genetic polymorphism associated with the human hepatic Mu-class GST has received a great deal of attention by workers in this field. However, whether the variations observed in the expression of these forms leads to changes in the susceptibility of an individual to cellular and genetic damage caused by electrophilic compounds is as yet unclear. There are reports which indicate that a lack of expression of the hepatic Mu-class GST increases an individuals sensitivity to the cancer-inducing or cancer-promoting compounds in tobacco smoke (Seidegard *et al.*, 1986, 1991) and may cause an increase susceptibility to adenocarcinomas of the colon and stomach (Strange *et al.*, 1991). However, by contrast, others have reported a similar distribution of GST $\mu/\psi$ in a large number of carcinoma patients compared to that found in a control population (Peters *et al.*, 1990). In addition, Zhong *et al.* (1991) have observed a positive correlation between the expression of Mu-class GST in patients with adenocarcinoma of the lung.

Several groups of workers have studied the influence that the GST Mu polymorphism has on the induction of cytogenetic damage. One such investigation demonstrated that lymphocytes obtained from GST μ deficient individuals are more sensitive to the induction of sister chromatid exchanges produced by *trans*-stilbene oxide than those obtained from individuals who expressed GST μ (Wiencke *et al.*, 1990). In another study, designed to investigate the consequences of the GST Mu polymorphism, Lui *et al.* (1991) attempted to determine the role of GST μ in modulating the formation of benzo(a)pyrene- and aflatoxin $B_1$-derived DNA adducts. Interestingly, although the presence of GST μ in hepatic cytosol did not reduce the formation of benzo(a)pyrene-derived adducts, aflatoxin $B_1$-DNA binding was inhibited to a greater extent by the presence of cytosol which contained this GST. These workers have, therefore, suggested that the hepatic Mu-class enzymes may be a susceptibility marker for aflatoxin $B_1$-related liver cancer.

The data presented in this thesis has shown that, in addition to the hepatic Mu-class GST subunits, at least two structurally and immunologically related Mu-class subunits are expressed in human skeletal muscle and testicular tissue. In order to establish the nature of the protective roles that these distinct Mu-class GST forms may perform a number of factors need to be determined. For example, it would be important to establish the catalytic activities of these GST towards a range of potentially harmful endogenous and xenobiotic electrophilic compounds. The work presented here has indicated that these enzymes may be involved in the detoxification of certain by-products of lipid peroxidation, in that they possess a high GSH conjugating activity towards 4-hydroxyalkenals, together with a relitively high peroxidase activity towards cumene hydroperoxide. However, it would also be of interest to determine the catalytic efficiency of these Mu-class subunits towards known mutagenic and carcinogenic compounds, such as the epoxides *trans*-stilbene oxide and benzo(a)pyrene-7,8-diol-9,10-epoxide. Furthermore, factors which influence the levels of expression of these Mu-class forms in particular tissues need to be considered. Throughout this project no evidence of genetic polymorphism in the expression of either the $N_2$ or the $N_3$ subunit in human skeletal muscle was observed. Other investigators have also reported no evidence of polymorphism in the expression of the extra-hepatic Mu-class isoenzymes GST4 (Suzuki *et al.*, 1987) and brain/testis μ (Campbell *et al.*, 1990). However, it is worth noting that inter-individual variations were observed in the levels of expression of the $N_2$ and the $N_3$ subunits in muscle specimens. Unfortunately, relitively little is known about the factors which control the differential expression of GST genes. It is of interest, however, that the 5′ flanking regions of the rat

$Yb_1$ and $Yb_2$ subunit genes are quite distinct, which suggests that unique mechanisms may regulate the expression of the individual Mu-class GST genes (Morton *et al.*, 1990).

The Theta-class GST which catalyse the conjugation of GSH to reactive sulphate esters formed from hydroxymethylarenes, such as 5-hydroxymethylchrysene, appear to play an important role in the prevention of carcinogenesis caused by these environmental pollutants. The fact that GST activity provides protection against this class of compound was first demonstrated when it was shown that GSH conjugation, mediated by GST present in rat hepatic cytosol, inactivates both 5-hydroxymethylchrysene sulphate and 7,12-dihydroxymethylbenz(a)anthracene sulphate and inhibits the formation of DNA adducts (Okuda *et al.*, 1986; Watabe *et al.*, 1987). Hiratsuka *et al.* (1990) subsequently purified a GST from rat liver which possesses catalytic activity towards a range of sulphate esters, including 1-menaphthyl sulphate. It is important to note that the level of hydroxysteroid sulphotransferase activity may also influence whether a particular tissue is target for carcinogenicity caused by hydroxymethylarenes. For example, rat liver which has a high level of hydroxysteroid sulphotransferase activity (Singer, 1985; Okuda *et al.*, 1989) is not a target for hydroxymethylarenes, whereas these compound show potent carcinogenicity to rat skin (Cavalieri *et al.*, 1979).

The work undertaken in this thesis has shown that human liver also possesses at least one GST isoenzyme with GSH conjugating activity towards reactive sulphate esters. An important area of further investigation would be to determine the tissue specificity and levels of expression of this novel human GST. It would also be of value to establish whether human Theta-class GST are subject to polymorphic expression.

# PART V

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# PART VI

# PUBLICATIONS

**Publications**

The following papers were published as a result of the work carried out for this thesis:-

Hussey A.J., Kerr L.A., Cronshaw A.D., Harrison D.J., & Hayes J.D. (1991). Variation in the expression of Mu-class glutathione S-transferase isoenzymes from human skeletal muscle. Evidence for the existence of heterodimers. Biochem J. **273**, 323-332.

Hussey A.J., & Hayes J.D. (1992). Characterization of a human class Theta glutathione S-transferase with activity towards 1-menaphthyl sulphate. Biochem J. (in press).

# Variation in the expression of Mu-class glutathione *S*-transferase isoenzymes from human skeletal muscle

## Evidence for the existence of heterodimers

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The cytosolic glutathione *S*-transferases (GST) from human skeletal muscle were purified by a combination of affinity chromatography and anion-exchange chromatography followed by either chromatofocusing or hydroxyapatite chromatography. Pi-class and Mu-class GST, but not Alpha-class GST, were isolated from muscle. In addition to a Pi-class GST subunit, which exists as a homodimer, this tissue also contains a total of three distinct neutral-type Mu-class GST subunits, which hybridize to form homodimers or heterodimers. The neutral-type subunits are referred to as $N_1$–$N_3$ and are defined by the decreasing isoelectric points of the homodimers; GST $N_1N_1$, $N_2N_2$ and $N_3N_3$ have estimated pI values of 6.1, 5.3 and < 5.0 respectively. SDS/PAGE showed that $N_1$, $N_2$ and $N_3$ have $M_r$ values of 26700, 26000 and 26300 respectively. The $N_1$, $N_2$ and $N_3$ subunits are catalytically distinct, with $N_1$ possessing a high activity for *trans*-4-phenylbut-3-en-2-one and $N_2$ having high activity with 1,2-dichloro-4-nitrobenzene. In skeletal muscle the expression of the $N_1$ subunit, but not of $N_2$ and $N_3$ subunits, was found to differ from specimen to specimen. The $N_1$ subunit was absent from about 50% of samples examined, and the purification results from two different specimens are presented to illustrate this inter-individual variation. Skeletal muscle from one individual (M1), which did not express $N_1$, contained only GST $N_2N_2$, $N_2N_3$ and $\pi$, whereas the second sample examined (M2) contained GST $N_1N_2$, $N_2N_2$ and $N_2N_3$ as well as GST $\pi$. *N*-Terminal amino acid sequence analysis supported the electrophoretic evidence that the $N_2$ subunit in GST $N_1N_2$, $N_2N_2$ and $N_2N_3$ represents the same polypeptide. The peptides obtained from CNBr digests of $N_2$ were subjected separately to automated amino acid sequencing, and the results indicate that $N_2$ is distinct but closely related to the protein encoded by the human Mu-class cDNA clone $GTH_4$ [DeJong, Chang, Whang-Peng, Knutsen & Tu (1988) Nucleic Acids Res. **16**, 8541–8554]. GST $N_2N_2$ is probably identical with GST 4 [Board, Suzuki & Shaw (1988) Biochim. Biophys. Acta **953**, 214–217], as over the 24 *N*-terminal residues of GST 4 there is complete identity between the two enzymes. Our data suggest that the GST 1 and GST 4 loci are part of the same multi-gene family.

## INTRODUCTION

The glutathione *S*-transferases (GST), a complex group of isoenzymes, function as a part of the phase II drug metabolism response, which provides protection against xenobiotics (Chasseaud, 1979; Ketterer, 1988; Pickett & Lu, 1989). The mammalian cytosolic enzymes are dimeric and can be divided into three classes, which are distinguishable by their physical, structural, catalytic and immunological characteristics (Mannervik, 1985; Hayes & Mantle, 1986*a*). In man these classes were originally described as the basic, near-neutral and acidic forms on the basis of the isoelectric points of the enzymes, but recently these have become more commonly referred to as Alpha-class, Mu-class and Pi-class GST respectively (Ålin *et al.*, 1985). Other workers, who have taken a genetic approach to the classification of these enzymes, have numbered the human GST according to their gene loci, using evidence obtained from zymogram analysis (Board, 1981; Strange *et al.*, 1984). This approach has given rise to the designation GST 1, GST 2 and GST 3, which are the loci that encode enzymes of the Mu-class, Alpha-class and Pi-class GST respectively.

In man, as in other species, the GST in liver have been the most extensively studied. Three hepatic Alpha-class enzymes formed by the combination of two structurally and immunologically distinct subunits termed $B_1$ and $B_2$ have been identified (Stockman *et al.*, 1985, 1987; Hayes *et al.*, 1989). The $B_1$ and $B_2$ subunits are the products of two independent genes (Rhoads *et al.*, 1987) encoded by the GST 2-1 and GST 2-2 loci (Board, 1981, 1990) respectively. Kamisaka *et al.* (1975) originally described five cationic forms of GST ($\alpha$, $\beta$, $\gamma$, $\delta$ and $\epsilon$) in human liver. Subsequent studies (Stockman *et al.*, 1985) have shown that the homodimeric enzyme $B_1B_1$ represents GST $\epsilon$ and the heterodimer $B_1B_2$ is equivalent to GST $\delta$. The relationship of $B_2B_2$ to these enzymes is less clear; this homodimer probably represents $\alpha$, $\beta$ and $\gamma$, three GST forms that may have arisen, during purification, from autoxidation of a single gene product (for further discussion see Hayes *et al.*, 1989).

The Mu-class GST in human liver are represented by two well-characterized enzymes termed $\mu$ (Warholm *et al.*, 1983) and $\psi$ (Hussey *et al.*, 1986; Singh *et al.*, 1987; Hayes, 1989). These homodimeric proteins are highly homologous (Hayes, 1989), and it has been suggested that they represent allelic variants encoded by the GST 1 locus, which was earlier identified by Board (1981) and Strange *et al.* (1984). The existence of a heterodimeric form

Abbreviation used: GST, glutathione *S*-transferase(s).

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of these enzymes has been described (Faulder *et al.*, 1987); however, so far it has not been purified from human tissue. Between 40 and 45% of individuals fail to express either of these Mu-class GST (Strange *et al.*, 1984; Hussey *et al.*, 1987), and it has been suggested that those lacking $\mu$ or $\psi$, which are the most efficient GST in the detoxification of mutagenic epoxides, may be more susceptible to lung cancer (Seidegård *et al.*, 1986, 1990).

The Pi-class GST isolated from placenta (Howie *et al.*, 1988) is the most thoroughly characterized extrahepatic enzyme. This enzyme is not expressed in hepatocytes, but is found in biliary epithelium, lung and erythrocytes. The available evidence suggests that these Pi-class enzymes, usually termed $\pi$, are the products of a single gene (Cowell *et al.*, 1988; Morrow *et al.*, 1989) encoded at the GST 3 locus.

The existence of further extrahepatic GST was first revealed by means of zymogram analysis (Laisney *et al.*, 1984). These enzymes, predominantly expressed in skeletal muscle, heart and brain, which were thought to be the products of additional gene loci termed GST 4, GST 5 and GST 6 (Suzuki *et al.*, 1987), are poorly characterized, and the molecular basis for the existence of these further enzyme forms (i.e. GST 4–6) is unclear. Board *et al.* (1988) have purified an enzyme from human skeletal muscle, called GST 4, that is immunologically related to GST $\mu$ and has *N*-terminal amino acid sequence homology with GST $\mu$. Others (Singh *et al.*, 1988) have isolated novel GST from skeletal muscle with *N*-terminal sequences either identical with or closely related to those of GST $\mu$ or GST 4.

In the present study we have purified skeletal-muscle GST from several individuals to permit the isoenzymes present to be characterized and to determine whether these enzymes are subject to inter-individual variation.

## EXPERIMENTAL

### Materials

Chemicals used were all of analytical grade and were readily available commercially. The h.p.l.c. solvents were obtained from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K.

### Buffers

The pH values quoted are those determined at the temperature at which the buffers were used.

### Tissues

Human skeletal-muscle specimens M1 and M2 were obtained less than 16 h *post mortem* from an 84-year-old female and a 60-year-old male respectively. A macroscopic examination at autopsy indicated that there was no evidence of any musculoskeletal abnormality in these individuals. Tissue was obtained from both right and left psoas muscles and stored at −70 °C until used.

### Analytical methods

Protein concentrations were determined by the method of Bradford (1976) with the use of a centrifugal fast analyser. SDS/PAGE was carried out by the method of Laemmli (1970) as described elsewhere (Hayes & Mantle, 1986*b*). The resolving gel contained 12% (w/v) polyacrylamide and 0.32% (w/v) *NN'*-methylenebisacrylamide.

Isoelectric focusing was performed in an LKB Multiphor apparatus, as described by the manufacturer (LKB Produkter, Bromma, Sweden), with a broad-range gel (pH 3.5–9.5) in thin-layer 5% (w/v) polyacrylamide. The gel was calibrated with protein pI standards obtained from Pharmacia, Milton Keynes, Bucks., U.K. The method of Habig & Jakoby (1981) was employed for all substrates studied with the exception of cumene hydroperoxide. Peroxidase activity was measured by an adaptation of the method of Wendel (1981) at pH 7.6 with final concentrations of GSH, NADPH and glutathione reductase at 1.0 mM, 0.25 mM and 1 unit/ml respectively.

### Enzyme purification

Portions of frozen skeletal-muscle specimen M1 (375 g) and M2 (247 g) were allowed to thaw at room temperature, and 25% (w/v) homogenates were prepared in ice-cold 20 mM-Tris/HCl buffer, pH 7.8, containing 200 mM-NaCl and 0.5 mM-dithiothreitol (buffer A). Unless otherwise stated all subsequent steps were performed at 4 °C. The supernatants at 100000 ***g***, referred to as cytosol, were passed through plugs of glass-wool before dialysis for 24 h against two changes, each of 5 litres, of buffer A.

M1 and M2 cytosols were then subjected to affinity chromatography on columns (1.6 cm × 30 cm) of *S*-hexylglutathione–Sepharose 6B equilibrated in buffer A. After a washing, with about 800 ml of buffer A, and elution by 5 mM-*S*-hexylglutathione in the same buffer, the bound material was dialysed for 24 h against two changes, each of 2 litres, of 20 mM-Tris/HCl buffer, pH 8.4, containing 0.5 mM-dithiothreitol (buffer B).

Partial resolution of the GST isoenzymes present in the affinity-purified material was achieved by the use of anion-exchange chromatography on Mono Q HR 5/5 with the integrated Pharmacia f.p.l.c. system. This procedure was carried out at room temperature. The Mono Q columns were equilibrated with buffer B (pH 8.0 at 20 °C) at 0.75 ml/min and developed in two stages with linear salt gradients of 0–0.15 M-NaCl in buffer B followed immediately by 0.15–1.0 M-NaCl in buffer B. Fractions eluted from the Mono Q column that contained protein were combined, seven peaks from M1 (P1–P7) and five peaks from M2 (P1, P3 and P5–P7), and analysed by SDS/PAGE before further purification.

The material designated P1, obtained from M2, was dialysed against two changes, each of 2 litres, of 25 mM-Bistris/iminodiacetic acid buffer, pH 7.1, before being chromatofocused, at 20 °C, in the pH range 7–4 on a Mono P HR 5/20 f.p.l.c. column (Pharmacia). Four protein-containing peaks were resolved by this method (P1-A, P1-B, P1-C and P1-D).

The GST activity present in peak P3 from Mono Q, purified from M1 and M2, was resolved into two peaks (P3-A and P3-B) by hydroxyapatite chromatography with a Waters h.p.l.c. system (Waters Associates, Northwich, Cheshire, U.K.). The material present in peak P3 was first dialysed against two changes, each of 2 litres, of 10 mM-sodium phosphate buffer, pH 7.0, containing 0.5 mM-dithiothreitol before application at room temperature to a Bio-Gel HPHT column (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.). A 10–350 mM-sodium phosphate gradient, at pH 7.0, was employed to develop the column at a flow rate of 0.5 ml/min.

### Immunoblotting

Antisera against purified human GST were raised in New Zealand White rabbits as described previously (Hayes & Mantle, 1986*a*). Western blotting was performed by the method of Towbin *et al.* (1979).

### Reverse-phase h.p.l.c.

This was carried out as described previously (Hayes *et al.*, 1989), with a Waters h.p.l.c. system with a $\mu$Bondapak $C_{18}$ column (Millipore, Harrow, Middx., U.K.). Purified skeletal-muscle GST subunits were resolved with linear gradients of 40–55% (v/v) acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid.

### CNBr cleavage

A portion (1.0 mg) of GST isoenzyme P3-B, purified under

reducing conditions, was dialysed extensively against 10 mM-ammonium acetate buffer, pH 7.0, before being freeze-dried. The freeze-dried protein was then subjected to CNBr cleavage as described elsewhere (Hayes *et al.*, 1989). The freeze-dried peptides were redissolved in 1.5 ml of aq. 0.1% (v/v) trifluoroacetic acid and resolved by reverse-phase h.p.l.c. on the μBondapak $C_{18}$ column with a 0–70% (v/v) acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid.

### Amino acid sequencing

Purified skeletal-muscle subunits and the fragments obtained from CNBr cleavage of isoenzyme P3-B were checked for purity on an Applied Biosystems 130 A Microbore Separation System (Applied Biosystems, Warrington, Cheshire, U.K.) before being subjected to automated sequencing on an Applied Biosystems 477A instrument with a 120A on-line phenylthiohydantoin analyser. For a complete description of these methods see Hayes *et al.* (1989).

## RESULTS

### Variability in the expression of GST isoenzymes in human skeletal muscle

Analyses of affinity-purified muscle GST by anion-exchange chromatography yielded profiles that differed from specimen to specimen (see Figs. 1*a* and 1*c*). Material from skeletal-muscle specimen M1 was resolved by Mono Q into seven protein-containing pools, two of which (P3 and P6) contained the major GST forms. SDS/PAGE showed that peaks P1 and P2, which did not have GST activity with 1-chloro-2,4-dinitrobenzene as substrate, both contained a single band with a subunit $M_r$ of 28500 (Fig. 1*b*). P3, the first major peak, contained the 28500-$M_r$ polypeptide together with bands that co-migrated with the human placental 24800-$M_r$ GST π standard and with a 26000-$M_r$ standard. The minor peaks P4 and P5 contained polypeptides of the same electrophoretic mobility as that of peak P3; however, peak P5 contained an additional band with an estimated subunit $M_r$ of 22500, which probably represents glyoxylase I (Hayes, 1988). The second major peak (P6) yielded two electrophoretic bands, one of which co-migrated with the 26000-$M_r$ standard and a second diffuse band with a subunit $M_r$ of 26300. This 26300-$M_r$ band was also observed in peak P7, as was the 22500-$M_r$ polypeptide tentatively identified as glyoxylase I.

By contrast, when the affinity-purified GST pool from skeletal-muscle specimen M2 was subjected to the same anion-exchange chromatography step only five peaks were resolved by Mono Q (Fig. 1*c*). The most significant difference between the elution profile of specimen M2 and that obtained from specimen M1 was the increased size of the first peak, P1, and the fact that, unlike peak P1 from the M1 specimen, this pool contained GST activity.

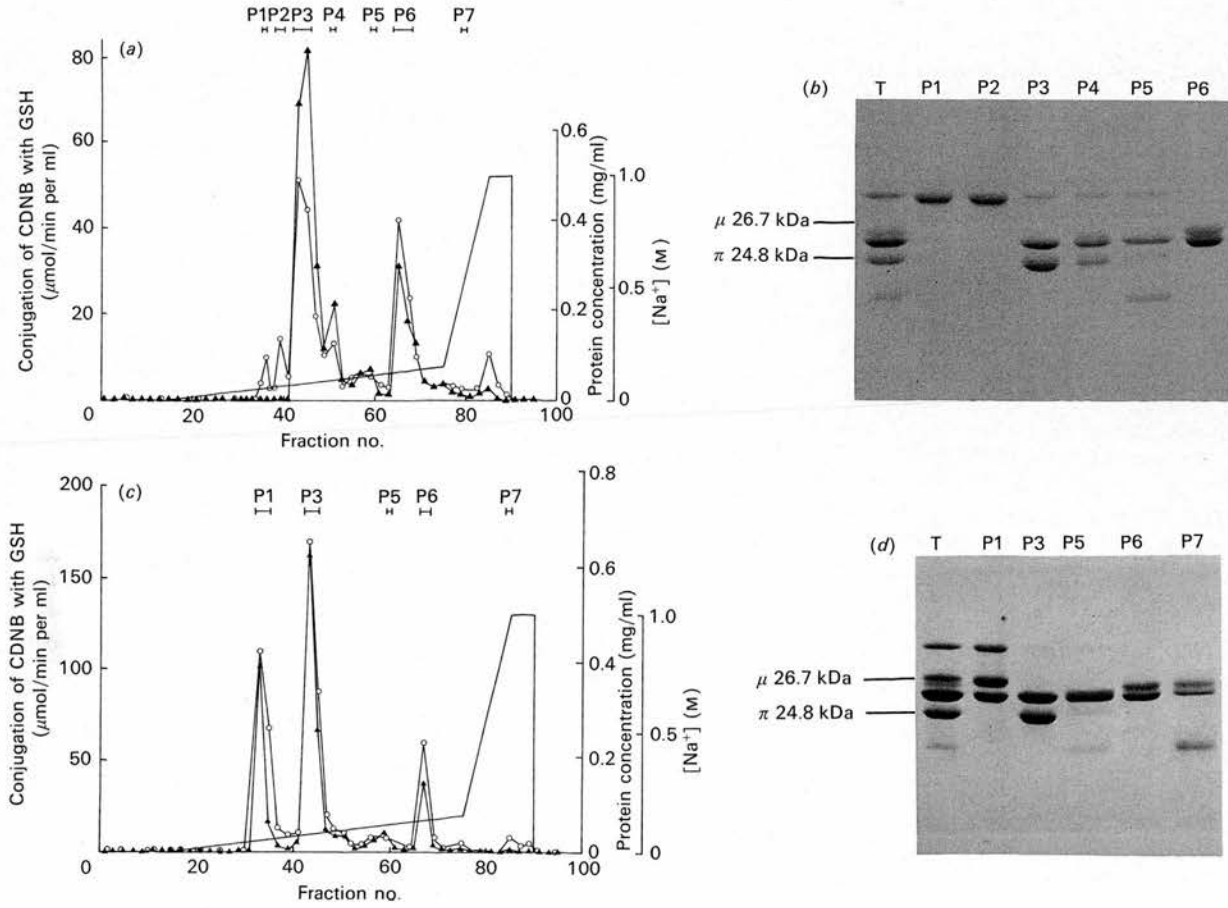


**Fig. 1. Resolution of human skeletal-muscle GST by anion-exchange chromatography and SDS/PAGE analysis of individual peaks**

*S*-Hexylglutathione-affinity-purified GST were resolved by anion-exchange chromatography on Mono Q as described in the text. A 0–1.0 M-NaCl gradient was established in two steps as shown by the straight lines. Transferase activity with 1-chloro-2,4-dinitrobenzene (CDNB) (▲) and protein concentration (○) were measured. Panels (*a*) and (*c*) show elution profiles obtained from material purified from specimens M1 and M2 respectively. The pooled fractions, indicated by the horizontal bars, were combined and subjected to electrophoretic analysis as shown in panels (*b*) and (*d*). SDS/PAGE was performed in a 12% (w/v) resolving gel. The positions of the human GST markers μ ($M_r$ 26700) and π ($M_r$ 24800), which were run in parallel, are indicated. The first lanes were loaded with total *S*-hexylglutathione-affinity-purified material (T; 6 μg). The following seven lanes in panel (*b*) and five lanes in panel (*d*) contained the combined fractions from Mono Q (4 μg).

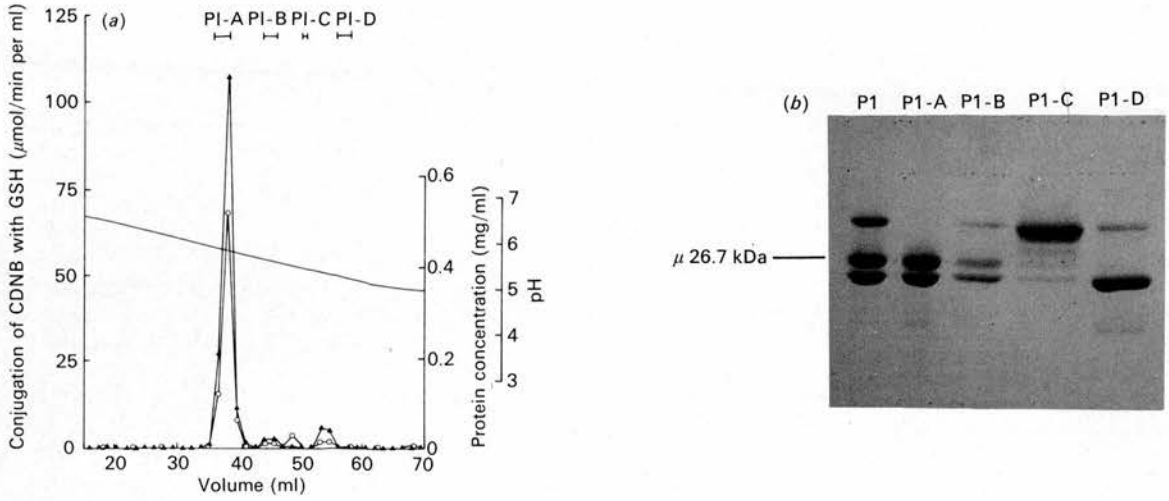


**Fig. 2. Chromatofocusing of partially purified skeletal-muscle GST and SDS/PAGE analysis of pooled fractions**

Skeletal-muscle GST activity, from specimen M2, purified by *S*-hexylglutathione affinity chromatography and anion-exchange chromatography on Mono Q (P1), was resolved by chromatofocusing on Mono P as described in the text (panel *a*). Transferase activity with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (▲) and protein concentration (○) were measured, and the pH was monitored (——). Fractions were combined, as indicated by the horizontal bars, and the subunit composition was analysed by SDS/PAGE (panel *b*). The first lane was loaded with the material applied to the chromatofocusing column (P1; 4 μg). The following lanes contained the pools obtained from Mono P, P1-A (4 μg), P1-B (1.5 μg), P1-C (4 μg) and P1-D (3 μg), as shown. The mobility of the human GST marker μ ($M_r$ 26700), which was run in parallel, is indicated.

When examined by SDS/PAGE (Fig. 1*d*), three subunit bands were observed; the largest was the 28500-$M_r$ polypeptide, the second band co-migrated with the human hepatic GST μ standard ($M_r$ 26700) and a third subunit co-migrated with the 26000-$M_r$ standard. Apart from the absence of the polypeptide of $M_r$ 28500, peaks P3, P5, P6 and P7 appeared to have the same subunit compositions as the corresponding peaks on the elution profile obtained from specimen M1. The additional subunit, present only in specimen M2, that co-migrated with GST μ ($M_r$ 26700) was also clearly visible when the affinity-purified total GST pool from specimen M2 was analysed by SDS/PAGE (T in Fig. 1*d*). Hence specimen M1 expressed GST subunits of $M_r$ 24800, 26000 and 26300 whereas specimen M2 expressed not only these three polypeptides but also a GST subunit of $M_r$ 26700.

The GST in muscle specimen M2 that was eluted from Mono Q in peak P1 were purified further by chromatofocusing on Mono P. This column resolved the material recovered in peak P1 into one major peak, P1-A, and three minor peaks, P1-B, P1-C and P1-D (Fig. 2*a*). The protein-containing fractions were combined and their subunit compositions examined by SDS/PAGE (Fig. 2*b*). Peak P1-A, which was eluted from the chromatofocusing column at pH 5.9, contained the two subunits that co-migrated during SDS/PAGE with the liver GST standards of $M_r$ 26000 and 26700. Electrophoresis showed that peak P1-B also contained these two subunits of $M_r$ 26000 and 26700 as well as the polypeptide with $M_r$ 28500. P1-C, the only peak that did not have GST activity, contained the contaminant polypeptide of $M_r$ 28500. Peak P1-D contained trace amounts of the contaminant and the GST subunit with $M_r$ 26000.

Hydroxyapatite h.p.l.c., a technique that separates the human hepatic GST according to their class (Hussey *et al.*, 1986), was employed to resolve the different subunit forms present in peak P3 from both muscle specimens. The profile obtained when material from specimen M2 was applied to this column is shown in Fig. 3(*a*). The first peak, which was eluted on the early part of the phosphate gradient, indicated the presence of Pi-class GST, and the second peak, which was eluted half-way along the gradient, indicated the presence of Mu-class subunits. Analysis by SDS/PAGE (Fig. 3*b*) confirmed that the first peak (P3-A) contained the subunit with the same mobility as GST π, whereas the second peak (P3-B) contained the subunit with an estimated $M_r$ of 26000. Peak P3-B possibly, therefore, contains Mu-class subunits with a greater anodal mobility during SDS/PAGE ($M_r$ 26000) than that of the Mu-class GST μ/ψ isoenzymes of human liver ($M_r$ 26700). The results of the purification of the GST isoenzymes of skeletal muscle are summarized in Table 1.

### Identification and characterization of three Mu-class isoenzymes and a Pi-class isoenzyme from human skeletal muscle

Upon analytical isoelectric focusing the GST pools P1-A, P3-A, P3-B and P6 focused into single bands, thereby confirming their purity. Electrophoretic analysis therefore indicates that peaks P3-A and P3-B probably contain homodimeric GST whereas peaks P1-A and P6 contain heterodimeric proteins. Western-blot analysis revealed that the components of peaks P1-A, P3-B and P6 cross-reacted with antisera raised against GST μ but not with antisera raised against either Alpha-class or Pi-class GST. By contrast, the component of peak P3-A was found to cross-react with antibodies to Pi-class GST but not with other antibodies against other GST. These immunochemical data suggest that peaks P1-A, P3-B and P6 contain Mu-class subunits and that peak P3-A contains Pi-class subunits. Together the immunochemical and electrophoretic data suggested the presence of three neutral-type Mu-class GST subunits in human muscle. By designating these as $N_1$, $N_2$ and $N_3$ (according to their apparent pI values) we were able to use a more systematic designation for the muscle enzymes that reflects their quaternary structures. Table 2 summarizes the physical and immunological characteristics of the skeletal-muscle GST; the hepatic GST μ is also included for comparison.

The specific activities of the muscle isoenzymes with a range of substrates are shown in Table 3. All of the enzymes purified from skeletal muscle have high activity with 1-chloro-2,4-dinitrobenzene as a substrate. However, when 1,2-dichloro-4-nitrobenzene or *trans*-4-phenylbut-3-en-2-one was employed considerably different patterns of activity were observed for the

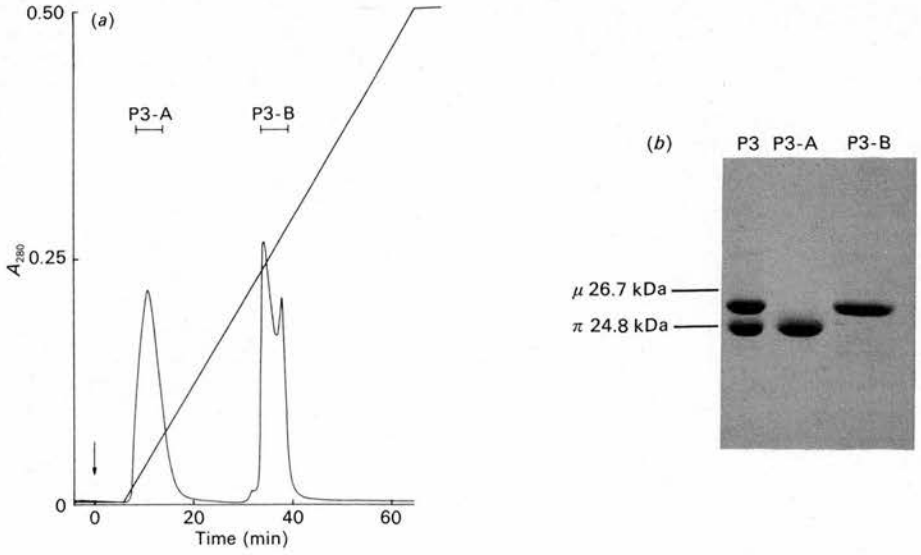


**Fig. 3. Hydroxyapatite chromatography of partially purified skeletal-muscle GST isoenzymes and SDS/PAGE of resulting peaks**

Enzymes purified from skeletal-muscle specimen M1 by *S*-hexylglutathione affinity chromatography and anion-exchange chromatography on Mono Q (P3) were further resolved by chromatography on Bio-Gel HPHT as described in the text (panel *a*). The column was developed with a linear 10–350 mM-sodium phosphate gradient, indicated by the straight line, and the $A_{280}$ was monitored (——). SDS/PAGE analysis of the combined fractions, indicated by the horizontal bars, is shown in panel (*b*). The mobilities of the human GST markers $\mu$ ($M_r$ 26700) and $\pi$ ($M_r$ 24800) are indicated. The first lane contained the material applied to the hydroxyapatite h.p.l.c. column (P3; 4 $\mu$g). The following lanes were loaded with the combined fractions, P3-A (4 $\mu$g) and P3-B (4 $\mu$g), as shown.

skeletal-muscle enzymes. The homodimeric Mu-class GST $N_2N_2$ (peak P3-B) and the heterodimeric Mu-class GST $N_2N_3$ (peak P6) both had activity with 1,2-dichloro-4-nitrobenzene but no detectable activity with *trans*-4-phenylbut-3-en-2-one, unlike the liver enzyme GST $\mu$, where the converse is true. The heterodimeric Mu-class GST $N_1N_2$ (peak P1-A), however, had activity with both of these substrates. GST $\mu$ and GST $N_1N_2$ were the only Mu-class enzymes to have activity with cumene hydroperoxide. The Pi-class skeletal-muscle enzyme (peak P3-A), like the placental enzyme GST $\pi$, had a relatively high activity with ethacrynic acid, but neither GST $N_1N_2$ nor GST $N_2N_2$ nor GST $N_2N_3$ was as active with this substrate.

### Subunit separation and *N*-terminal sequence analysis

Before sequence analysis, reverse-phase h.p.l.c. was employed in an attempt to prepare separately the individual subunits present in the skeletal-muscle GST isoenzymes. Unfortunately, the $N_1$ and $N_2$ subunits could not be resolved on the $\mu$Bondapak $C_{18}$ column, but the $N_3$ subunit was resolved from these two Mu-class polypeptides by reverse-phase h.p.l.c. This technique was also used to purify further GST $N_2N_2$ (peak P3-B) by removing small residual quantities of the 28500-$M_r$ polypeptide that contaminated certain preparations (Fig. 4). The elution of the two subunits present in GST $N_2N_3$ (peak P6) from the $\mu$Bondapak

**Table 1. Purification of human skeletal-muscle GST isoenzymes**

For experimental details see the text. GST activity was measured with 1-chloro-2,4-dinitrobenzene at 37 °C.

| | Skeletal-muscle specimen M1 | | | Skeletal-muscle specimen M2 | | |
|---|---|---|---|---|---|---|
| Fraction | Total protein (mg) | Total activity ($\mu$mol/min) | Specific activity ($\mu$mol/min per mg) | Total protein (mg) | Total activity ($\mu$mol/min) | Specific activity ($\mu$mol/min per mg) |
| Cytosol | 9007 | 5419 | 0.60 | 9742 | 4817 | 0.49 |
| Affinity-chromatography pool | 19.8 | 2245 | 77.8 | 19.4 | 3817 | 196.7 |
| Mono Q f.p.l.c. pools: | | | | | | |
| P1 | – | – | – | 3.1 | 631.2 | 201.0 |
| P3 | 3.9 | 933.0 | 240.5 | 4.1 | 1160 | 283.6 |
| P6 | 2.7 | 253.0 | 92.3 | 0.9 | 163.0 | 187.4 |
| Mono P f.p.l.c. pool: | | | | | | |
| P1-A | – | – | – | 1.12 | 152.0 | 135.7 |
| HPHT h.p.l.c. pools: | | | | | | |
| P3-A | 0.66 | 74.0 | 112.5 | 0.73 | 87.6 | 120.0 |
| P3-B | 0.59 | 100.8 | 171.1 | 1.14 | 243.0 | 213.1 |

**Table 2. Physical and immunochemical properties of skeletal-muscle GST isoenzymes**

Preparations of antisera were as described in Hayes *et al.* (1983) (GST $B_1B_1$), Hayes (1989) (subunit $N_1$ or GST $\mu$) and Howie *et al.* (1988) (GST $\pi$). Anti-(subunit $N_2$) IgG was prepared as described in the Experimental section using $N_2$ subunits, purified by reverse-phase h.p.l.c., as immunogen.

| Enzyme | Preparation designation | Subunit $M_r$ | pI value | Cross-reactivity with antisera against: $B_1B_1$ | $N_1$ | $N_2$ | $\pi$ |
|---|---|---|---|---|---|---|---|
| $N_1N_1$ ($\mu$)* | – | 26700 | 6.10 | – | +++ | +++ | – |
| $N_1N_2$ | P1-A | 26700 | 5.65 | – | +++ | +++ | – |
| | | 26000 | | – | ++ | +++ | – |
| $N_2N_2$ | P3-B | 26000 | 5.30 | – | ++ | +++ | – |
| $N_2N_3$ | P6 | 26000 | 5.00 | – | ++ | +++ | – |
| | | 26300 | | – | + | – | – |
| $\pi$ | P3-A | 24700 | 4.70 | – | – | – | +++ |

* Data from Hayes (1989)

**Table 3. Specific activities of skeletal-muscle GST isoenzymes**

For experimental details see the text. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; tPBO, *trans*-4-phenylbut-3-en-2-one; EA, ethacrynic acid; CuOOH, cumene hydroperoxide.

| Enzyme | Preparation designation | Specific activity ($\mu$mol/min per mg at 37 °C) CDNB | DCNB | tPBO | EA | CuOOH |
|---|---|---|---|---|---|---|
| $N_1N_1$ ($\mu$)* | – | 261 | 0 | 0.22 | 0.08 | 0.22 |
| $N_1N_2$ | P1-A | 136±3 | 2.67±0.04 | 0.26±0.04 | 0.41±0.13 | 0.17±0.01 |
| $N_2N_2$ | P3-B | 171±7 | 1.91±0.32 | 0 | 0.33±0.04 | 0 |
| $N_2N_3$ | P6 | 92±3 | 1.61±0.2 | 0 | 0.27±0.01 | 0 |
| $\pi$ | P3-A | 113±1 | 0.25±0.06 | 0 | 0.78±0.01 | 0 |

* Data from Hayes (1989).

$C_{18}$ column is also shown in Fig. 4. The major peak, which was eluted at between 37 and 41 min, contained a polypeptide of $M_r$ 26000, whereas the two minor peaks, which were eluted at 50 min and 56 min, both contained subunits of $M_r$ 26300. SDS/PAGE analysis of the GST subunits that were isolated by this technique is shown in Fig. 5.

*N*-Terminal sequence analysis was undertaken to help establish the relationship between the muscle GST and provide evidence that the polypeptide of $M_r$ 26000 that was found in the different Mu-class enzymes, and that we have designated $N_2$, is indeed a common subunit. The results of these analyses are shown in Fig. 6. The subunits of $M_r$ 26000 present in the GST $N_2N_2$ homodimer

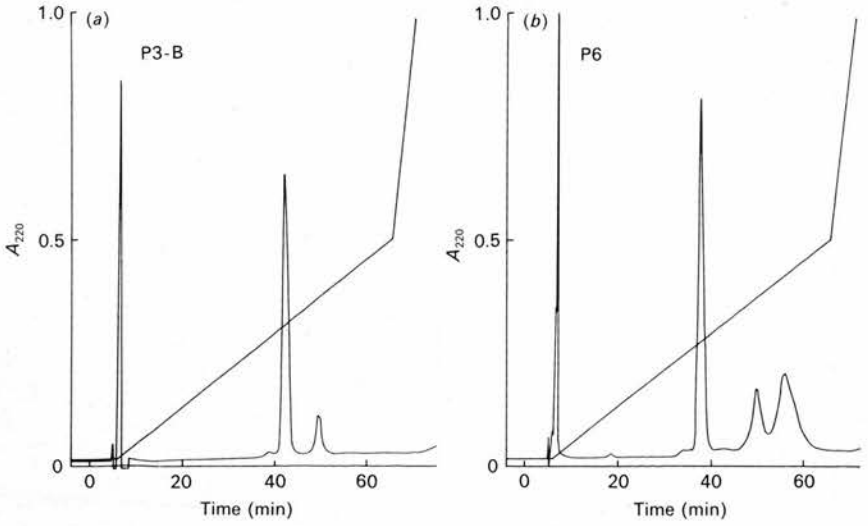


**Fig. 4. Reverse-phase h.p.l.c. of human skeletal-muscle GST subunits**

Skeletal-muscle GST pools P3-B (*a*) and P6 (*b*) were subjected to reverse-phase h.p.l.c. on a Waters $\mu$Bondapak $C_{18}$ column (10 $\mu$m particle size; 0.39 cm × 30 cm). The column was developed with a 40–70% (v/v) acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid in two stages as shown by the straight lines. The $A_{220}$ of the eluate was monitored continuously (——).

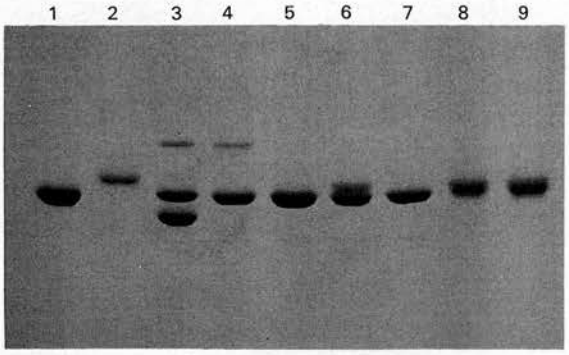


**Fig. 5. SDS/PAGE analysis of Mu-Class subunits isolated from human skeletal muscle**

SDS/PAGE was performed by using the method of Laemmli (1970). The resolving gel contained 12% (w/v) polyacrylamide and 0.32% (w/v) *NN'*-methylenebisacrylamide. The GST subunits purified on reverse-phase h.p.l.c. were analysed as follows. Lane 1 contained the subunit marker $B_1B_2$ ($M_r$ 26000) and lane 2 contained the subunit marker $\mu$ ($M_r$ 26700). Lane 3 was loaded with pool P3 obtained after anion-exchange chromatography on Mono Q of affinity-purified material (4 $\mu$g). Lane 4 contained pool P3-B purified on hydroxyapatite h.p.l.c. (4 $\mu$g). Lane 5 contained the GST subunit $N_2$ isolated from pool P3-B after reverse-phase h.p.l.c. Lane 6 was loaded with pool P6 purified by affinity chromatography and anion-exchange chromatography on Mono Q (4 $\mu$g), lane 7 contained the GST subunit $N_2$ isolated from P6, and lanes 8 and 9 contained the $N_3$ subunits resolved on reverse-phase h.p.l.c. from P6.

and the GST $N_2N_3$ heterodimer were shown to have identical *N*-terminal sequences over the first ten residues. Although the subunits in GST $N_1N_2$ could not be separated on the $\mu$Bondapak $C_{18}$ column it was possible to interpret the sequence data obtained from the heterodimeric protein over most of the residues analysed. The direct sequencing of GST $N_1N_2$ yielded unambiguous assignments from cycles 1, 2, 4, 5, 6, 7, 9, 10, 11, 12, 13 and 14, but at residues 3, 8 and 15 the Sequenator found two amino acids, namely threonine or isoleucine, asparagine or aspartic acid and serine or alanine. When these sequences were compared with those of human Mu-class enzymes described in the literature, it was apparent that one sequence could be attributed to that of human liver GST $\mu$ or GST $\psi$ (Ålin *et al.*, 1985; Hayes, 1989) and the other was identical with the 24 residues published for the skeletal-muscle enzyme GST 4 (Board *et al.*, 1988). These data suggest that the subunit of $M_r$ 26000, designated $N_2$, in GST $N_1N_2$, $N_2N_2$ and $N_2N_3$ represents the same polypeptide. The $N_3$ subunit that was purified by reverse-phase h.p.l.c. was found to possess a blocked *N*-terminus and could not be sequenced directly.

## Comparison of the amino acid sequences of human Mu-class GST isoenzymes

The protein sequence information obtained from the muscle Mu-class enzyme GST $N_2N_2$ has enabled the comparison of 52% of its primary structure with that of other human Mu-class enzymes reported in the literature. Four of the peptides, CNBr-2, CNBr-5, CNBr-6 and CNBr-7, were subjected to automated amino acid sequencing. In Fig. 7 the amino acid sequences obtained from these fragments, together with the *N*-terminal sequence of subunit $N_2$ (peptide CNBr-1), are shown aligned with the homologous sequences deduced from the human hepatic cDNA clone $GTH_4$, reported by DeJong *et al.* (1988), and the sequences deduced from the human Mu-class genomic clones, GST mu2 and GST mu3, described by Taylor *et al.* (1990).

Comparison of the *N*-terminal sequence of GST $N_2N_2$ (peptide CNBr-1) with that of the $GTH_4$ clone reveals the three amino acid differences found at positions 3, 8 and 15 predicted from the *N*-terminal sequence analysis of GST $\mu$ and GST $\psi$ discussed above. This is followed by a region of complete identity between the two sequences from residue 16 to residue 38. The peptide CNBr-2, which was eluted from the $\mu$Bondapak $C_{18}$ column at 44.5 min, was subjected to 45 automated Edman degradation cycles, allowing identification of residues 34–78. Over this region

| Enzyme | Pool | Subunit(s) examined | Residue 1 5 10 15 20 |
|---|---|---|---|
| $N_1N_1$ ($\mu$)* | | $N_1$ | P M I L G Y W D I R G L A H A I R L L L E Y T D |
| $N_1N_2$ | P1-A | $N_1+N_2$ | P M I/T L G Y W D/N I R G L A H A/S |
| $N_2N_2$ | P3-B | $N_2$ | P M T L G Y W N I R |
| $N_2N_3$ | P6 | $N_2$ | P M T L G Y W N I R G L A H S I R L L L E Y T D |
| $N_2N_3$† | P6 | $N_3$ | – |
| GST 4‡ | | | P M T L G Y W N I R G L A H S I R L L L E Y T D |

**Fig. 6. *N*-Terminal amino acid sequences of GST from human skeletal muscle**

Purified skeletal-muscle GST enzymes $N_1N_2$, $N_2N_2$ and $N_2N_3$ were subjected to automated *N*-terminal amino acid sequencing as described in the text. *Data from Hayes (1989). †*N*-Terminus blocked. ‡Data from Board *et al.* (1988).

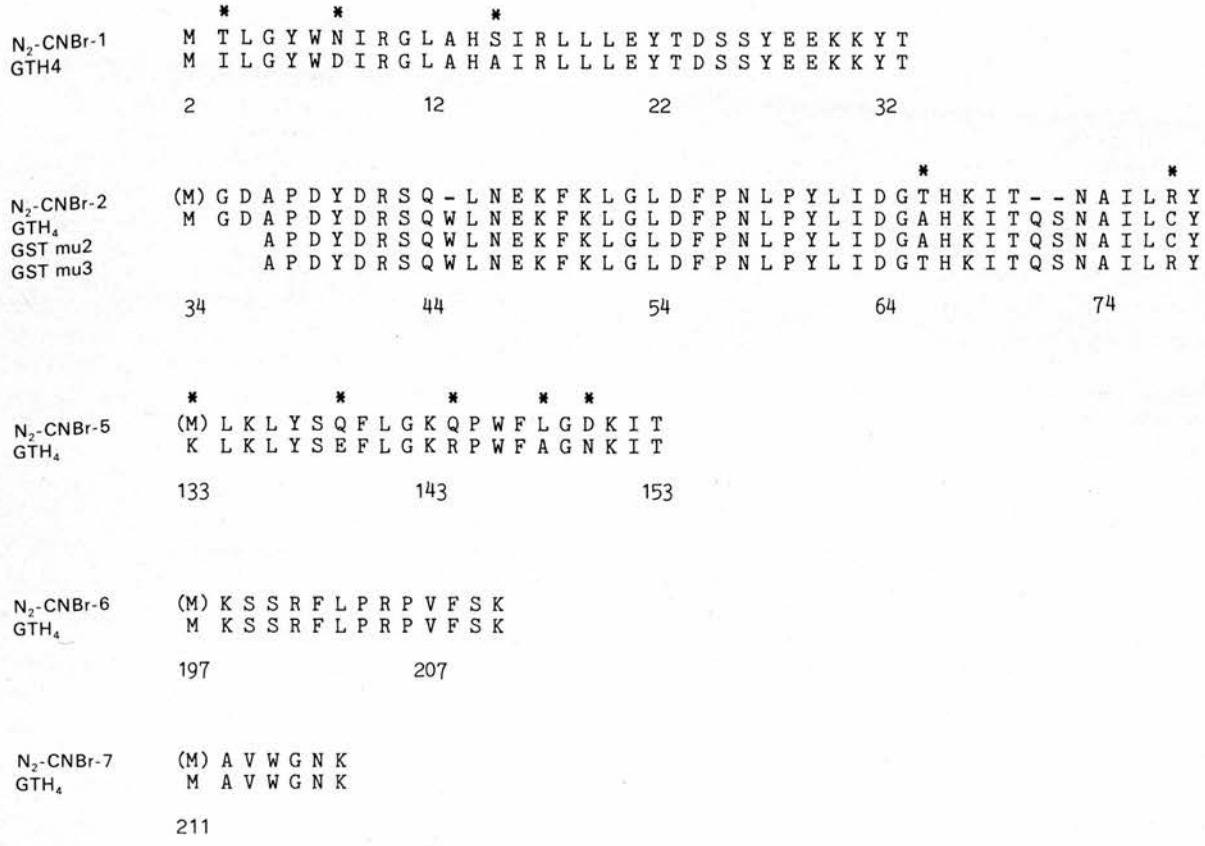


**Fig. 7. Comparison of the primary structure of the $N_2$ subunit with other human Mu-class GST**

The *N*-terminal amino acid sequence (CNBr-1) and the sequences of the CNBr-derived peptides from subunit $N_2$ are aligned for comparison with the homologous sequences deduced from the cDNA $GTH_4$ (DeJong *et al.*, 1988) and the available homologous sequences deduced from the genomic clones GST mu2 and GST mu3 (Taylor *et al.*, 1990). Putative methionine residues are in parentheses and a dash indicates where no residue could be identified with confidence. An asterisk (*) indicates amino acid residues that differ between the sequences.

of the protein amino acid sequence information on the genomic clones GST mu2 and GST mu3 is available for comparison with subunit $N_2$. Amino acid differences were apparent between the sequence of the $N_2$-subunit peptide CNBr-2 and those of the proteins encoded by clones $GTH_4$ and GST mu2. At position 66 the $N_2$ subunit contains threonine whereas the proteins encoded by clones $GTH_4$ and GST mu2 contain alanine, and at position 77 the $N_2$ subunit possesses arginine whereas the proteins encoded by clones $GTH_4$ and GST mu2 both contain cysteine. However, when the sequence of $N_2$-subunit peptide CNBr-2 is compared with that of the protein encoded by clone GST mu3 it is apparent that the two sequences correspond exactly over the region where sequence data on this genomic clone are available (i.e. residues 37–78).

Residues 133–153 of subunit $N_2$ were identified by sequencing the peptide, CNBr-5, that was eluted at 51 min from the $\mu$Bondapak $C_{18}$ column. Five amino acid substitutions were present between the sequence of $N_2$-subunit peptide CNBr-5 and that of the protein encoded by clone $GTH_4$. At position 133 a methionine residue, which has provided a CNBr-cleavage site, can be assigned to subunit $N_2$, whereas the protein encoded by clone $GTH_4$ contains a lysine residue. The other four amino acid changes between $N_2$-subunit peptide CNBr-5 and the protein encoded by clone $GTH_4$, at positions 139, 144, 148 and 150, were as follows: glutamine to glutamic acid, glutamine to arginine, leucine to alanine and aspartic acid to asparagine respectively. $N_2$-subunit peptides CNBr-6 and CNBr-7, which were eluted at 30 min and 23 min from the reverse-phase column, contained residues 198–210 and residues 212–217 respectively. No differences were apparent between this *C*-terminal region of the $N_2$ subunit and the homologous region of the protein encoded by clone $GTH_4$.

## DISCUSSION

This paper describes the purification of a total of four GST isoenzymes from human skeletal muscle. Three of these were Mu-class GST and the remaining enzyme was a Pi-class GST. No Alpha-class enzymes were isolated from any of the specimens of human muscle. The Mu-class, or neutral, enzymes were shown to comprise three distinct subunits, which we have designated $N_1$, $N_2$ and $N_3$ according to their decreasing pI values. These subunits can hybridize, and we have described the muscle GST by their quaternary structure as $N_1N_2$, $N_2N_2$ and $N_2N_3$. During the present study a $N_1N_3$ heterodimer has not been recovered, and although its existence seems probable we can offer no explanation for its absence from human muscle. The $N_1$ subunit was found in only about half of the muscle specimens examined, whereas $N_2$ and $N_3$ were recovered in all specimens. Because of the variation in the expression of $N_1$, the $N_1N_2$ heterodimer was absent from some of the enzyme preparations.

GST $N_1N_2$, $N_2N_2$ and $N_2N_3$ possess distinct catalytic properties. The $N_1N_2$ heterodimer was found to possess the greatest activity of all the muscle GST for *trans*-4-phenylbut-3-en-2-one, indicating that the $N_1$ subunit is responsible for catalysing its conjugation with GSH. All these GST were active with 1,2-

dichloro-4-nitrobenzene, which strongly implicates the $N_2$ subunit in the metabolism of this compound. No substrate was identified that could serve as a marker for the $N_3$ subunit. It may be noted that the substrate-specificities of the $N_1N_2$, $N_2N_2$ and $N_2N_3$ muscle enzymes are significantly different from the well-characterized hepatic Mu-class enzyme, GST $\mu$ (Table 3). When one examines the activities of these enzymes for 1,2-dichloro-4-nitrobenzene and *trans*-4-phenylbut-3-en-2-one, both diagnostic substrates for the Yb-type subunits in the rat, it is apparent that GST $N_2N_2$ has a similar substrate-specificity to that of the rat enzymes $Yb_1Yb_1$ and YnYn, whereas the substrate preference of GST $\mu$ is more closely similar to that of rat GST $Yb_2Yb_2$ (Hayes, 1986).

A modest decrease in the specific activity of the purified GST isoenzymes, with 1-chloro-2,4-dinitrobenzene as substrate, is apparent towards the end of the purification procedure (Table 1). Having examined the data from five skeletal-muscle preparations, we have observed a relatively large amount of variability in the specific activities obtained for the purified Mu-class isoenzymes GST $N_1N_2$ and GST $N_2N_2$, whereas the Pi-class enzyme has a specific activity that varies little from preparation to preparation. The basis for this observation is uncertain, but it does not appear to be due to proteolysis, as neither the $M_r$ nor the immunochemical properties of GST $N_1N_2$ or GST $N_2N_2$ change during the purification. It therefore seems probable that either an aging effect or the removal of an activator during purification is causing a loss in the enzymic activity of these Mu-class GST in certain preparations. In this context it is noteworthy that certain rat Mu-class GST are labile, and Kispert *et al.* (1989) have proposed that the thiol status of the cysteine residue at position 184 in rat subunit 11 (Yo) is responsible for this property in this particular GST.

The muscle Mu-class GST have different isoelectric points, and their pI values are helpful aids to the identification of the various isoenzymes; GST $N_1N_2$, $N_2N_2$ and $N_2N_3$ have isoelectric points of 5.65, 5.30 and 5.00 respectively. However, because of the closeness of these pI values it is unwise to identify GST solely on the basis of isoelectric-focusing results. The subunits that the Mu-class GST comprise can be resolved conveniently, and identified, by SDS/PAGE. With this method the $N_1$, $N_2$ and $N_3$ subunits have estimated $M_r$ values of 26700, 26000 and 26300 respectively. It is important to recognize that the $N_2$ subunit and the Alpha-class $B_1/B_2$ subunits co-migrate during SDS/PAGE and that it is therefore essential to use immunochemical methods to help discriminate between these subunits.

No cross-reactivity was observed between subunits $N_1$, $N_2$ and $N_3$ and antibodies against either the Alpha-class GST $B_1B_2$ or the Pi-class GST $\pi$. All the N-type subunits were found to cross-react with antiserum raised against hepatic GST $\mu$, and therefore, although the $N_2$ and $B_1/B_2$ subunits co-migrate during SDS/PAGE, they can be discriminated by Western-blot analysis. However, our data also suggest that the individual N-type subunits can be distinguished by immunochemistry, but that to achieve identification the choice of antiserum is important. The degree of cross-reactivity towards GST $\mu$ antibodies was found to vary from subunit to subunit. Whereas the $N_1$ subunit cross-reacted strongly with this antiserum, the $N_2$ subunit showed a significantly weaker reactivity, giving a moderate signal in the Western-blot assay and less than 10% cross-reactivity in the GST $\mu$ radioimmunoassay (A. F. Howie, A. J. Hussey, J. D. Hayes & G. J. Beckett, unpublished work). By contrast, the $N_3$ subunit was even less reactive than the $N_2$ subunit towards anti-(GST $\mu$) antibodies, yielding only a weak signal in the Western-blot assay. We have also raised antibodies against the $N_2$ subunit and found that, although these cross-react with the $N_1$ subunit, they do not exhibit reactivity with the $N_3$ subunit.

*N*-Terminal amino acid sequencing suggested that subunits $N_1$, $N_2$ and $N_3$ are genetically separate. Although we were able to analyse the purified $N_2$ and $N_3$ subunits directly, we were unable to resolve by reverse-phase h.p.l.c. the constituent subunits of GST $N_1N_2$ and therefore the sequence data for subunit $N_1$ were derived from the direct analysis of the $N_1N_2$ heterodimer. Comparison between subunits $N_1$ and $N_2$ showed differences at residues 3, 8 and 15; at these positions subunit $N_1$ appeared to contain isoleucine, aspartic acid and alanine whereas subunit $N_2$ was found to contain threonine, asparagine and serine. By contrast, the $N_3$ subunit possesses a blocked *N*-terminus, but unfortunately paucity of material prevented us from undertaking a preparative CNBr digest that could have allowed us to obtain sequence data.

Comparison of the *N*-terminal sequence data for subunit $N_1$ with the primary structure of the subunits of hepatic GST $\mu$ suggests that they are identical. This conclusion is supported by the immunochemical results, the observation that subunit $N_1$ has a high activity for *trans*-4-phenylbut-3-en-2-one and the inter-individual variability in the expression of the $N_1$ subunit. Inspection of the sequence data for the $N_2$ subunit compared with the cDNA clone encoding GST $\mu$ (i.e. $N_1N_1$) shows that they are genetically distinct; differences in primary structure were noted at residues 3, 8, 15, 66, 77, 133, 139, 144, 148 and 150. Moreover, comparison of these data for the $N_2$ subunit with the *N*-terminal sequence for GST-4 (Board *et al.*, 1988) suggests that subunits $N_2$ and GST-4 are probably the same. The amino acid sequence analysis of subunit $N_2$ also allowed comparison with the genomic human Mu-class clones of Taylor *et al.* (1990). This indicated that $N_2$ represents the subunit encoded by clone GST mu3. The fact that the $N_3$ subunit possesses a blocked *N*-terminus is itself an interesting observation, as in the rat the only Mu-class GST subunit that has a blocked *N*-terminus is Yo (Hayes, 1988; Kispert *et al.*, 1989), the $Yb_1$, $Yb_2$, $Yn_1$ and $Yn_2$ subunits having *N*-termini that are amenable to direct sequence analysis. It is therefore possible that subunit $N_3$ is the homologue of the rat Yo subunit. It is also worth noting that the Mu-class homodimer GST $\phi$ of pI 4.6, which Stockman & Hayes (1987) found in only one of 20 human liver specimens, may represent $N_3N_3$ as it also possessed a blocked *N*-terminus.

We have demonstrated the ability of the $N_2$ subunit to hybridize with other Mu-class subunits. The existence of a heterodimeric protein formed by the combination of the subunits encoded by the GST 1 locus (i.e.$N_1$) and the GST 4 locus (i.e. $N_2$) was first predicted, by using zymogram analysis, by Laisney *et al.* (1984). This finding, however, was not confirmed when similar studies were performed by Suzuki *et al.* (1987). The isolation and characterization, described in the present paper, of the $N_1N_2$ heterodimer supports the earlier work of Laisney *et al.* (1984), and the purification of the $N_2N_3$ heterodimer represents new information abut the multiplicity of the human Mu-class GST family. It is of interest that the chromosome mapping experiments performed by several workers have located human Mu-class genes on both chromosome 1 (DeJong *et al.*, 1988) and chromosome 3 (Islam *et al.*, 1989).

Two hepatic human Mu-class homodimers, GST $\mu$ and GST $\psi$, have been described (Hussey *et al.*, 1986; Hayes, 1989). These enzymes represent allelic variants and possess closely similar catalytic activities and identical *N*-terminal amino acid sequences (up to 50 residues), but differ in their isoelectric points, with GST $\mu$ and GST $\psi$ having pI values of 6.1 and 5.5 respectively. As GST $\mu/\psi$ appear to comprise $N_1$-type subunits, we propose that GST $\mu$ be designated $N_1^aN_1^a$ and GST $\psi$ be designated $N_1^bN_1^b$. On the basis of respective pI values of 6.1, 5.5 and 5.3 for $N_1^aN_1^a$, $N_1^bN_1^b$ and $N_2N_2$, it is probable that the $N_1N_2$ heterodimer of pI 5.65 described in the present study represents

$N_1{}^aN_2$; it is expected that the isoelectric point of the $N_1{}^bN_2$ heterodimer would be approx. 5.4. This difference in the pI value of the allelic variants of $N_1$ is an important reason why extreme caution should be exercised in identifying these enzymes solely by isoelectric focusing.

The acidic enzyme, peak 3-A, which was present in large quantities in skeletal muscle of all individuals included in the study, has similar physical and catalytic properties to those of the Pi-class enzyme found in human placenta. Other workers have also observed an enzyme, or enzymes, similar to the placental GST $\pi$ in skeletal muscle (Board *et al.*, 1988; Singh *et al.*, 1988). Awasthi and co-workers (Singh *et al.*, 1988) found two acidic GST isoenzymes, termed GST 4.8 and GST 4.5, that have identical *N*-terminal amino acid sequences to each other and to that of human placental GST $\mu$. One of these enzymes, GST 4.5, was present in only two of six skeletal muscles studied, and these workers therefore proposed the existence of a polymorphism at the GST 3 locus. Although we have not observed any difference in the Pi-class GST from different specimens of skeletal muscle, it should be recognized that hybridization *in situ* has localized the gene encoding Pi-class GST in man to both chromosomes 11 and 12 (Board *et al.*, 1989). The possibility therefore remains that two GST $\pi$ isoenzymes exist, but this question remains to be resolved. In this context, it is interesting that the amino acid sequence of fatty acid ethyl ester synthetase from human myocardium has been reported to be highly similar to, but not identical with, that of the placental GST $\pi$ (Bora *et al.*, 1989). On the basis of the data of Ålin *et al.* (1985) and Bora *et al.* (1989) it is apparent that over the 23 *N*-terminal residues of fatty acid ethyl ester synthetase and placental GST $\pi$ two amino acid differences exist at residues 1 (alanine→proline) and 15 (lysine→alanine). There is therefore evidence that two separate Pi-class GST are expressed in man, but it is unclear how these relate to the GST 4.5 and GST 4.8 forms described by Singh *et al.* (1988).

From the results presented here it is clear that there are at least one Pi-class and three Mu-class subunits expressed in skeletal muscle. Evidence has been provided that the three Mu-class subunits combine to form homodimeric or heterodimeric proteins and that one of these subunits is absent from the muscle of certain individuals. The characterization of the $N_1$, $N_2$ and $N_3$ subunits has provided a valuable insight to the molecular basis for the multiplicity of Mu-class GST in man.

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