5

THE IDENTIFICATION, PURIFICATION AND CHARACTERIZATION OF THE FETAL RAT LIVER GLUTATHIONE S-TRANSFERASE ISOENZYME Y_cY_{fetus}

by

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ABSTRACT

THE IDENTIFICATION, PURIFICATION AND CHARACTERIZATION OF THE FETAL RAT LIVER GLUTATHIONE S-TRANSFERASE ISOENZYME Y_cY_{fetus}

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This study has examined the expression of the glutathione S-transferases (GSH S-T) in fetal rat livers in order to provide more information about the role played by this important group of enzymes in the fetus. The study commenced with an examination of the subunit composition of adult and fetal rat liver GSH S-T using affinity chromatography followed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate. Adult livers contained four major GSH S-T subunits. An additional and previously unidentified subunit was detected in fetal livers. This subunit, which differed from that found in rat placenta, had a M_r of approximately 25 500. Densitometric measurements suggest that the newly detected subunit accounts for as much as 26% of the GSH S-T in fetal livers.

The novel fetal isoenzyme comprising this subunit was purified using a combination of affinity chromatography, carboxymethyl-cellulose column chromatography and chromatofocusing. The six major basic rat liver GSH S-T were purified for reference and comparative purposes. The fetal isoenzyme is composed of two non-identical subunits, namely, subunit Y_c (M_r 28 000) and the fetal subunit referred to as 'Y_{fetus}'. The enzyme which I have termed GSH S-transferase Y_cY_{fetus} has an isoelectric point of approximately 8.65 and has GSH S-T activity towards a number of substrates. Significantly, the fetal isoenzyme has one of the highest glutathione peroxidase activities yet described for the purified rat liver GSH S-T towards the model substrate, cumene hydroperoxide. Kinetic studies reveal that the fetal isoenzyme has a catalytic efficiency for the peroxide substrate which is four fold higher than that of the adult rat liver isoenzyme, GSH S-T Y_cY_c.

The in vitro effect of the GSH S-T substrate and teratogen, acrolein, on this fetal isoenzyme was investigated and compared with acrolein's effect on some of the adult rat liver GSH S-T isoenzymes in the standard 1-chloro-2,4-dinitrobenzene assay. Surprisingly, acrolein was identified as a non-competitive inhibitor of the GSH S-T. Exposure to acrolein in various guises could therefore result in inhibition of the fetal isoenzyme and its subsequent failure in inhibiting lipid peroxidation. Inhibitor studies were performed to look at the effect of acrolein, as well as other substrate and non-substrate ligands, on the glutathione peroxidase activity of GSH S-T Y_cY_{fetus} and Y_cY_c. The glutathione peroxidase activity of the fetal isoenzyme was far less susceptible to acrolein inhibition than the Y_cY_c isoenzyme and the fetal isoenzyme was found to retain significant glutathione peroxidase activity despite saturating concentrations of non-substrate ligand.

This study suggests that the fetal isoenzyme serves a specific function in protecting fetuses against the possible teratogenic effects of organic peroxides.

February, 1988.

TABLE OF CONTENTS

Page

PART A : INTRODUCTION AND LITERATURE REVIEW

PART B : EXPERIMENTAL

PART A

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The glutathione S-transferases (GSH S-T) (EC 2.5.1.18) are a family of multifunctional enzymes which play an important role in the metabolism of a wide variety of xenobiotics (foreign compounds). Xenobiotics are usually hydrophobic compounds and require conversion to water soluble forms in order to facilitate their excretion. This involves one or more of four chemical mechanisms, namely, oxidation, reduction, hydrolysis (Phase I reactions), and conjugation with endogenous substrates (Phase II reactions). The GSH S-T are responsible for phase II reactions in which a wide variety of electrophilic compounds, including many phase I biotransformation products, are conjugated to reduced glutathione (GSH).

Environmental xenobiotics, as well as those administered therapeutically, may be transmitted to the fetus in utero via the maternal circulation. Some xenobiotics are known to be toxic to the fetus. This toxicity has usually been attributed to the limited ability of the fetal enzyme systems to detoxify foreign compounds (Pelkonen, 1980). The enzymes and enzyme systems responsible for the inactivation of biologically active xenobiotics in the fetus have not been fully characterized and most enzyme studies to-date have been limited to the immediate pre- and postnatal periods. This is especially applicable to the fetal GSH S-T.

This study has examined the expression of the GSH S-T in fetal rat livers in order to provide more information about the role played by this important group of enzymes in the fetus. Since the GSH S-T are dimeric proteins, and the various monomers which associate noncovalently to make up these dimers presumably confer the observed differences in affinity which these enzymes exhibit towards various substrates, the study commenced with an examination of the subunit composition of fetal rat liver GSH S-T. This led to the identification and subsequent purification of a GSH S-T isoenzyme present only in fetal rat livers. The enzyme, which I have termed glutathione S-transferase Y_cY_{fetus}, has been characterised and its physico-chemical and immunological properties as well as its affinity for several standard substrates have been compared with those of the various adult rat liver GSH S-T. The implications of these studies and of the finding that GSH S-T Y_cY_{fetus} has one of the highest glutathione peroxidase activities yet described for the purified rat liver GSH S-T have been examined with particular reference to their possible role in the fetotoxicity of certain xenobiotics.

Thus this thesis will describe :

(i) the purification of the six major basic adult rat liver GSH S-T isoenzymes,

(ii) the identification of a GSH S-T isozyme in fetal rat livers which is absent from adult rat livers,

(iii) the purification and characterization of the novel fetal rat liver GSH S-T isoenzyme, Y_cY_{fetus},

(iv) the inhibition of the fetal and adult rat liver GSH S-T isoenzymes by the teratogen, acrolein, and

(v) kinetic and inhibitor studies relating to the glutathione peroxidase activity of GSH S-T Y_cY_{fetus} and Y_cY_c.

CHAPTER 1

LITERATURE REVIEW

1.1. THE GSH S-TRANSFERASES : HISTORY AND NOMENCLATURE

The soluble forms of GSH S-T are all dimeric proteins and as such, are composed of two noncovalently associated subunits. These subunits have similar molecular weights whether isolated from plants (Mozer et al, 1983) or man (Kamisaka et al, 1975a).

A further distinguishing feature of these enzymes is the occurence of multiple forms of the GSH S-T in an organism (Mannervik, 1985a). The identification of these multiple forms is based on their specific activities with various substrates, M_r of their subunits, isoelectric points, sensitivity to inhibitors, reaction with specific antibodies, peptide maps, and amino acid sequences.

The GSH S-T were first described by Booth et al in 1961. The first GSH S-T form(s) to be purified was a basic or cationic rat liver GSH S-T. This took place in three independent laboratories engaged respectively in investigating the binding of azodye carcinogens, cortisol metabolytes and organic anions to proteins in the cytosol of rat hepatocytes. In 1971 the three liver cytosolic proteins known as azodye carcinogen-binding protein (Ketterer et al, 1967), corticosteroid Binder I (Morey and Litwack, 1969) and Y protein (Levi et al, 1969), were identified as being a single protein, which was termed ligandin (Litwack et al, 1971). In 1973 Kaplowitz et al discovered that the ligand, sulphobromophthalein (BSP), which they were using to study hepatic organic anion transport, was conjugated to GSH in the presence of ligandin, and in 1974 Habig et al (1974a) identified ligandin as a GSH S-T enzyme. Bass et al (1977a) found that ligandin, which had previously been thought to be a homodimer composed of identical subunits, was in fact a mixture of homo- and hetero- dimers, and that the so-called "Y peak" of rat liver cytosol which bound organic anions contained at least three closely related subunits which they termed Y_a, Y_b and Y_c. This led to several studies in which the rat GSH S-T were shown to consist of various combinations of these and other closely related subunits.

This multiplicity of subunits has created difficulties in the nomenclature adopted for these enzymes. Originally, the classification of the GSH S-T was based on the substrate specificity of the enzymes (Boyland and Chasseaud, 1969), for example, GSH S-aryltransferase, GSH S-epoxidetransferase, GSH S-alkyltransferase, GSH S-alkenetransferase. Unfortunately, considerable overlapping of substrate specificity precluded the use of this method. When in 1973 Clark et al discovered that 1-chloro-2,4-dinitrobenzene (CDNB) was a universal substrate for all GSH S-T, an attempt was made at naming these proteins according to their elution from carboxymethyl-cellulose columns. Jakoby et al (1976) isolated 6 GSH S-T using this system which they termed AA, A, B, C, D and E, according to their reverse order of gradient elution. However the introduction of substrate affinity chromatography yielded several additional transferases which soon overwhelmed Jacoby's alphabetical system. Following the description by Bass et al (1977a) of the Y_a, Y_b and Y_c subunits, Mannervik and Jensson (1982) found the Y_b band to contain two major components Y_b and $Y_{b'}$. They drew up a nomenclature in which each subunit was named by a single letter of the alphabet. More recently a numerical system has been introduced in which the various subunits are given a number referring to the order in which they have been isolated and characterized (Jakoby et al, 1984). For example, Y_a, Y_c, Y_b and $Y_{b'}$ are termed subunits 1, 2, 3 and 4 respectively.

Difficulties in the nomenclature of the GSH S-T are compounded by the possible existence of variants in the primary structure of a subunit (Mannervik, 1985a). Amino acid sequences deduced from two cDNA clones of rat liver subunit Y_a have been shown to differ in 8 positions and C-terminal amino acid determinations of subunit Y_a have suggested similar limited heterogeneity (Beale et al, 1983).

Where possible, the nomenclature used in this thesis will be that of Bass et al (1977a). Table 1.1 lists the alternative nomenclature used for the various isoenzymes.

The human GSH S-T identified to date have been denoted by Greek letters (Mannervik, 1985a). However, the recent discovery that these may also be heterodimeric compounds (Soma et al, 1986) suggests the need for a new method by which they may be classified.

Table 1.1. **ALTERNATIVE NOMENCLATURE FOR THE RAT GSH S-TRANSFERASES***

GSH S-T	Alternative Nomenclature (References)									
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Y_aY_a	1-1	B	Ligandin	Ligandin	X	B_1	L_2			
Y_aY_c	1-2	B	Ligandin	B		B_2	BL			
Y_cY_c	2-2	AA				AA	B_2			
Y_bY_b	3-3	A				A	A_2		II	
$Y_bY_{b'}$	3-4	C				C	AC		I	
$Y_{b'}Y_{b'}$	4-4	D?#				D	C_2	X		
	5-5	E				E				
Y_tY_t	6-6									M_T
Y_pY_p	7-7(11)									

*Modified from Mannervik (1985a).
#GSH S-transferase D was not fully characterized by Jakoby et al.

References : (1) Jakoby et al (1984); (2) Jakoby et al (1976); (3) Litwack et al (1971); (4) Hayes et al (1981); (5) Scully and Mantle (1981); (6) Beale et al (1983); (7) Mannervik and Jensson (1982); (8) Friedberg et al (1983); (9) Askelof et al (1975); (10) Dierickx and De Beer (1981); (11) Meyer et al (1985).

1.2. FUNCTIONS OF THE GSH S-TRANSFERASES

1.2.1. Catalytic Functions

1.2.1.1. Exogenous Substrates

The GSH S-T are able to catalyze a number of reactions between GSH and the electrophilic centres of various xenobiotics. A feature of these enzymes is the possession of broad and overlapping second substrate specificities with any one GSH S-T catalyzing the conjugation of GSH with several different types of second substrate (Jakoby et al, 1976). This makes them an efficient armament against a vast array of xenobiotics.

Most of the exogenous substrates for GSH S-T are synthetic xenobiotics, or their phase I biotransformation products. Criteria used for detection of such substrates include enzyme catalyzed formation of GSH adducts in tissue preparations and bile, and the excretion of mercapturic acids in the urine. Work with purified enzymes has involved a more restricted range of substrates. Generally, electrophilic carbon (Boyland and Chasseaud, 1969; Jakoby, 1978) in various chemical guises is susceptible to one or more of the isoenzyme forms of the GSH S-T (Douglas et al, 1987). These include alkyl (Chasseaud, 1973) and aryl (Chari and Kozarich, 1981) halides and congeners (where the leaving groups may be chloride, iodide, bromide, nitrite or sulphate); epoxides and erene oxides (Hayakawa et al, 1975); or addition to alpha,beta-unsaturated carbonyl compounds (Habig et al, 1974b; Clifton and Kaplowitz, 1977) by the Michael reaction.

Less well studied catalytic reactions are those in which displacements at elements other than carbon occur, for example, nitrogen and sulfur may also be attacked. Organic nitrates are cleaved by GSH S-T catalyzed attack of GSH on electrophilic nitrogen (Keen et al, 1976), and attack at sulfur in organic thiocyanates (Keen et al, 1976) also occurs. Fig. 1.1 illustrates just a few of the many xenobiotics which serve as substrates for the GSH S-T (see review by Chasseaud, 1979).

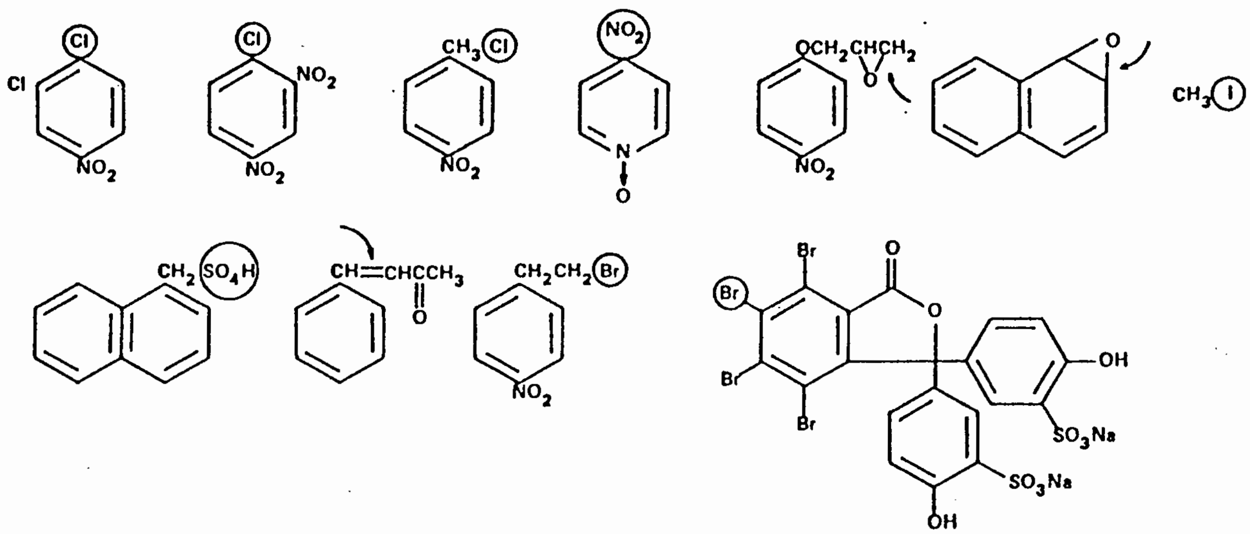
1.2.1.2. Endogenous Substrates

Little is currently known about the role played by the GSH S-T in the metabolism of endogenous substrates. A possible endogenous substrate is the quinone, dopaquinone, since a corresponding GSH conjugate has been identified in human malignant melanoma (Agrup et al, 1977)

Epoxides may constitute a group of possible substrates as endogenous compounds, as well as xenobiotics, may be metabolized to form epoxides and GSH conjugation plays a major role in their biotransformation (Mannervik, 1985a). Recent research has focused on the possible role of the GSH S-T in leukotriene synthesis. Here the epoxide derivative of arachidonic acid, leukotriene A_4, is conjugated with GSH to give leukotriene C_4 by hepatic and microsomal GSH S-T (Mannervik et al, 1984).

Sulphate esters arising in cellular metabolism may also act as substrates as arylalkyl sulphates such as benzyl sulphate have been shown to act as substrates for the GSH S-T (Gillham, 1971).

1 2 3 4 5 6 7



8 9 10 11

Fig. 1.1. Substrates in the GSH S-transferase catalyzed reaction with GSH. Leaving groups or sites of addition are indicated (From Jakoby et al, 1976).

1 : 1,2-Dichloro-4-nitrobenzene
2 : 1-Chloro-2,4-dinitrobenzene
3 : p-Nitrobenzyl chloride
4 : 4-Nitropyridine-N-oxide
5 : 1,2-Epoxy-3-(p-nitrophenoxy)propane
6 : 1,2-Naphthalene oxide
7 : Iodomethane
8 : 1-Menaphthyl sulphate
9 : trans-4-Phenyl-3-buten-2-one
10 : p-Nitrophenethyl bromide
11 : Sulphobromophthalein

Glutathione S-Transf. & Catalysis pg 189 - 211.

The formation of GSH conjugates both in vivo and in vitro suggest that reactive intermediates of estradiol-17beta and 2-hydroxy-estradiol-17beta (Elce and Harris, 1971) may be substrates for the GSH S-T (Mannervik, 1985a). Cholesterol alpha-oxide, which is a product of lipid peroxidation, has also been identified as a substrate for the GSH S-T (Meyer and Ketterer, 1982).

A recent study has also shown that 4-hydroxynon-2-enal, a major product of peroxidative degradation of polyunsaturated fatty acids, is an active substrate for rat cytosolic GSH S-T (Alin et al, 1985a).

The GSH S-T are able to catalyze ketosteroid isomerization in which GSH serves a true co-enzyme role by not being consumed (Benson and Talalay, 1976; Benson et al, 1977). No intermediate conjugate for this reaction has been found. It has been postulated that ketosteroid isomeraze activity may be implicated in the step in bile acid synthesis involving isomerization from cholest-5-en-3-one to cholest-4-en-3-one (Ketterer, 1986).

The GSH S-T may play a role in the formation of prostaglandins from postaglandin endoperoxides. A preparation of sheep lung with GSH S-T activity has been shown to catalyze the formation of prostaglandins D_2, E_2 and F_{2alpha} from prostaglandin H_2 (Nugteren and Christ-Hazelhof, 1973; Christ-Hazelhof et al, 1976). A GSH dependent enzyme (possibly a GSH S-T) from bovine vesicular gland microsomes, has similarly been shown to catalyze the conversion of prostaglandin H_1 to prostaglandin E_1 (Ogino et al, 1977). However, the exact role of the GSH S-T in prostaglandin metabolism remains unknown.

The GSH S-T also possess selenium-independent GSH peroxidase activity for organic hydroperoxides (Prohaska and Ganther, 1977) and an important biological function of the GSH S-T may be the protection of membranes from lipid peroxidation (see Section 1.11. for a review of the GSH S-T and lipid peroxidation).

1.2.2. Binding Functions

1.2.2.1. Non-Covalent Binding

The GSH S-T have been shown to bind, non-covalently, to numerous lipophilic substrate and non-substrate ligands (Ketley et al, 1975; Tipping et al, 1976a). It was this binding property that gave ligandin (GSH S-T B) its name.

The list of non-substrate ligands is extensive including :

(i) organic anionic dyes (e.g. indocyanine green, BSP, rose bengal) (Kamisaka et al, 1975b; Jakoby, 1978);

(ii) antibiotics (e.g. penicillin, cephalothin, tetracycline, chloramphenicol) (Kornguth et al, 1974; Kamisaka et al, 1975b);

(iii) bile salts and bile acids (e.g. chenodeoxycholate, cholate, lithocholate) (Hayes and Mantle, 1986; Takikawa et al, 1986);

(iv) fatty acids (e.g. palmitate) (Tipping et al, 1976a);

(v) radiographic contrast media (e.g. iodipamide) (Kamisaka et al, 1975b);

(vi) haem proteins and their derivatives (e.g. bilirubin, haemin, uroporphyrin I, uroporphyrin III, haematoporphyrin) (Kamisaka et al, 1975b; Tipping et al, 1976b);

(vii) thyroid hormones (e.g. tri-iodothyronine and thyroxine) (Kamisaka et al, 1975b);

(viii) steroid hormones and their metabolites (e.g. cortisol, corticosterone, testosterone, oestrone, diethylstilboestrol) (Kamisaka et al, 1975b; Tipping et al, 1976a; Homma et al, 1986).

The list of substrate ligands includes, sulphobromophthalein (Pabst et al, 1974; Jakobson et al, 1979), benzyl chloride (Pabst et al, 1974), trans-4-phenyl-3-buten-2-one (Pabst et al, 1974), epoxides (Hayakawa et al, 1975) and many GSH conjugates (Pabst et al, 1974; Jakobson et al, 1979; Ridgewell and Abdel-Monem, 1987).

The binding function of the GSH S-T is thought to be necessary to prevent reflux of these lipophiles from the hepatocyte into the blood (Wolkoff et al, 1979) i.e. the GSH S-T may play an important role as intracellular storage and/or transport proteins.

The binding of these ligands results in inhibition of the GSH S-T (Pabst et al, 1974; Hayakawa et al, 1975; Ketley et al, 1975; Chasseaud, 1979; Boyer et al, 1984; Ong and Clark, 1986; Ridgewell and Abdel-Monem, 1987) and will be covered in more detail in Section 1.3.3.

Ridgewell and Abdel-Monem (1987) have recently looked at GSH S-T catalyzed conjugation of alkyl halides in vitro and confirmed that product inhibition occurs at low GSH concentrations. Excessive exposure to xenobiotics could therefore result in accumulation of GSH conjugates in a particular tissue in vivo, leading to reaction product-inhibition of the GSH S-T and their subsequent failure in detoxification.

1.2.2.2. Covalent Binding

In addition to non-covalent binding, the GSH S-T also bind certain ligands covalently. These include activated carcinogenic metabolites of 3-methylcholanthrene and azo-dye carcinogen metabolites (Ketterer and Christodoulides, 1969; Singer and Litwack, 1971). This covalent binding is a suicidal process that results in inactivation of the enzyme and is thought to be an additional protective mechanism for the cell against the harmful effects of xenobiotics. Acrylamide (Mukhtar et al, 1981) and hypolipidemic drugs such as clofibrate (Foliot et al, 1986) are xenobiotics known to inhibit hepatic GSH S-T activity following in vivo administration. However, inactivation of the enzymes by one xenobiotic may only serve to expose the cell to the harmful effects of another. An example of this may be seen after the intraperitoneal administration of the radiosensitizer misonidazole which inhibits hepatic GSH S-T as well as selenium-dependent and selenium-independent GSH peroxidase activities (Kumar and Weiss, 1986). It is postulated that this may lead to elevation of intracellular peroxide levels, contributing to the radiosensitizing effect and/or toxicity of misonidazole.

1.3. THE GSH S-TRANSFERASES : MECHANISM OF ACTION

1.3.1. Catalytic Function

The reactions catalyzed by the GSH S-T generally consist of the nucleophilic attack of GSH on various electrophiles with the subsequent formation of mercapturic acid (Boyland and Chasseaud, 1969), the classical urinary excretion product of xenobiotics. Fig. 1.2 shows a schematic representation of the suggested biochemical pathway for the biotransformation of an electrophilic xenobiotic. The electrophile (I) may be any compound capable of reacting with the free thiol group (-SH) of glutathione (II) and forming a stable conjugate (III). The reactive group -X, represents both the chemical groups that are eliminated such as halogenides, and those that are modified without elimination such as epoxides. The glutathione conjugate (III) is degraded in two steps to a corresponding cysteine conjugate (V). Acetylation of the conjugate (V) with acetylcoenzyme-A results in its conversion to a mercapturic acid (VI). Alternatively, the cysteine conjugate may be cleaved to yield a mercaptan by elimination of pyruvate and ammonia. The resulting thiol group of the mercaptan may be either glucuronosylated with uridinediphosphoglucuronate to a thioglucuronide, or methylated with S-adenosylmethionine to a methylthio conjugate which may be oxidized sequentially to a methylsulfinyl and a methylsulfonyl derivative.

Uncertainty exists over the precise chemical mechanism of enzyme catalysis. It has been suggested that the GSH S-T increase the nucleophilicity of the thiol group of GSH (Jakoby, 1978). The enhanced

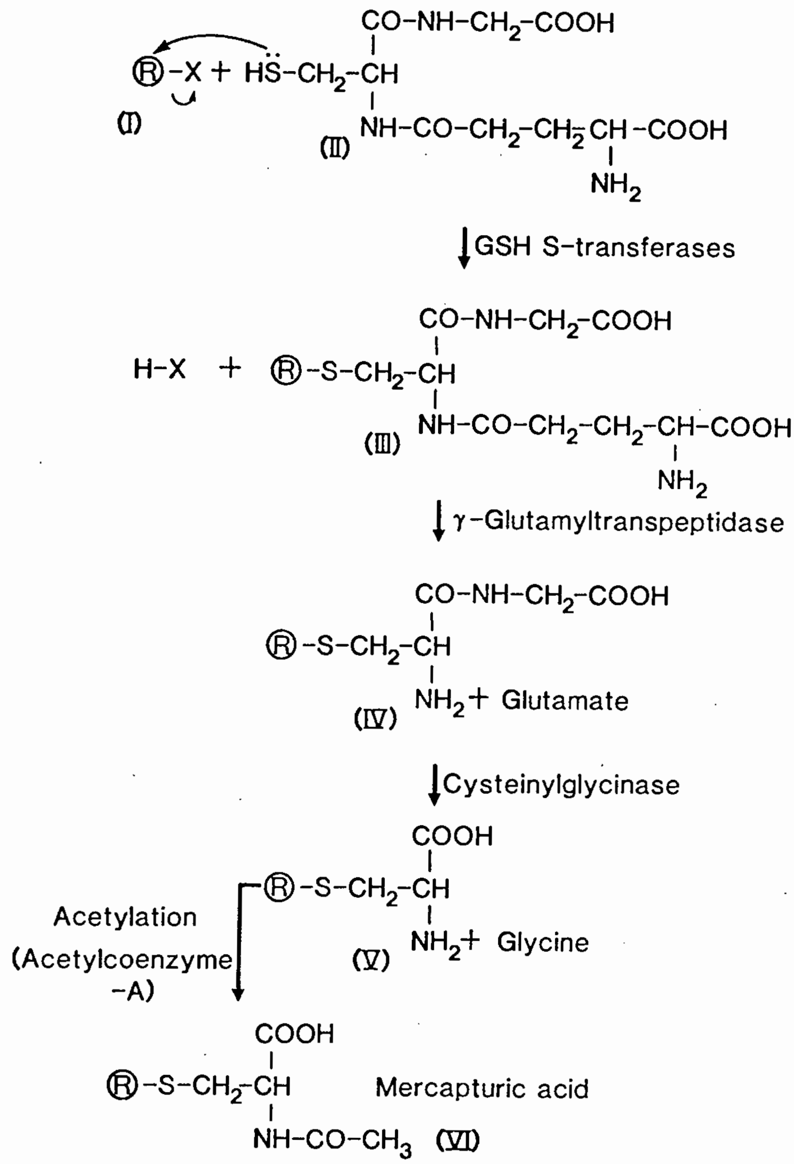


Fig. 1.2. **Schematic representation of the suggested biochemical pathway for the biotransformation of an electrophilic xenobiotic as catalyzed by the GSH S-transferases (Modified from Mannervik, 1985a).**

nucleophilicity has been ascribed to an active-site base (Mannervik et al, 1978). One mechanism proposed to achieve this was that the enzymes may lower the pK_a of the SH group ($pK_a = 9.2$). Attempts to measure the pK_a of bound GSH spectrophotometrically proved to be technically difficult but there was no net proton change (no uptake or release) on binding of GSH to GSH S-T B (Jakoby, 1978). Thus the question of facilitation of ionization is still unanswered. It has also been proposed that the enzyme-bound electrophile may provide a hydrophobic environment which aids reactivity (Keen et al, 1976).

1.3.2. Role of Various Subunits

There is considerable evidence that the various subunits of the GSH S-T influence the ability of the intact enzymes to catalyze their various reactions and to bind substrate and non-substrate ligands. For example, in the rat the homodimer comprising subunit Y_a is associated with high isomeraze activity with delta^5-androstene-3,17-dione and high affinity ligand binding for haem and bilirubin (Jakoby and Habig, 1980; Alin et al, 1985b). Y_c has high activity with ethacrynic acid (Meyer et al, 1985), Y_b has high activity with 1,2-dichloro-4-nitrobenzene (DCNB) and $Y_{b'}$ with trans-4-phenyl-3-buten-2-one (Alin et al, 1985b). Subunits Y_a and Y_c have GSH peroxidase activity while subunits Y_b and $Y_{b'}$ have very little peroxidase activity (Mannervik, 1985a). The heterodimers have properties that are generally intermediate between those of the respective homodimers (Mannervik, 1985a).

1.3.3. Enzyme Activity and Non-Substrate Ligand Binding

The non-substrate ligands of the GSH S-T act as inhibitors of enzyme activity. Early studies indicated that the inhibition of GSH S-T Y_aY_c by a variety of non-substrate ligands was competitive, suggesting that the non-substrate binding and catalytic sites were similar (Ketley et al, 1975). However, more recent studies suggest that the catalytic and binding sites are different (Bhargava et al, 1978; Vander Jagt et al, 1982), although some non-substrate ligands may bind at the active site (Vander Jagt et al, 1982).

Boyer et al (Boyer et al, 1984) examined the relationship between GSH S-T catalytic activity and the binding of non-substrate ligands. Using indocyanine green, biliverdin and chenodeoxycholate as inhibitors of activity of GSH S-T Y_aY_a, Y_aY_c and Y_cY_c, they found the activities of Y_aY_c and Y_cY_c were inhibited at low (pH 6.0) but not high (pH 8.0) pH values. Between these values intermediate degrees of inhibition were observed. In contrast, transferase Y_aY_a was inhibited by at least 80% at all pH values. Failure of the enzyme to bind the inhibitor could not account for these differences. Thus those enzymes containing the Y_c subunit are able to act simultaneously as both enzymes and binding proteins. This indicates that some forms of the GSH S-T assume different conformations after the binding of non-substrate ligands which determines whether or not enzymatic activity will be lost. The conformation is dependent on the presence of non-substrate ligand and on the pH. Clinically it is possible that during cholestasis, when intracellular concentrations of non-substrate ligands are high, some of the isoenzymes may retain significant activity and protect the liver from toxic xenobiotic electrophiles. Alternatively, changes in intracellular pH could have detrimental effects on enzyme function (Boyer, 1985).

1.3.4. Non-Substrate Ligand Binding Site

Uncertainty prevails over the non-substrate ligand binding site for these enzymes. Bilirubin binds to transferases Y_aY_c and to Y_aY_a with high affinity (2 moles of bilirubin/mole of GSH S-T) but not to Y_cY_c, which led Bhargava et al (1980a; 1980b) to believe that the high affinity site is on subunit Y_a. The same phenomenon was observed for the binding of lithocholate by the transferases (Hayes et al, 1981). However, others have found a single high affinity binding site for bilirubin on GSH S-T Y_aY_a (Carne et al, 1979; Maruyama et al, 1984) suggesting that the binding site does not reside on a particular subunit but is formed by an interaction between the subunits. Boyer (1986) has recently examined the non-substrate ligand binding site of GSH S-T Y_aY_a and Y_aY_c with bilirubin-Woodward's reagent K which labels the ligand binding site covalently. He found that the label blocked the non-substrate ligand binding site of the two enzymes but not the catalytic site, and the divalent reagent labelled the subunits of transferase Y_aY_c equally, again suggesting that the single high affinity bilirubin-binding site present on this enzyme is formed by an interaction between the subunits rather than residing on a specific subunit.

1.4. CHARACTERIZATION OF INDIVIDUAL GSH S-TRANSFERASES

The following section will describe the characteristics of the major rat GSH S-T isoenzymes purified to date. Although the human transferases do not form the basis of this thesis, they will receive brief mention to elicit a clearer understanding of the literature review on the ontogeny of the GSH S-T (Section 1.8.).

1.4.1. Rat GSH S-Transferases : Liver

The cytosolic rat liver GSH S-T are probably the most extensively studied isoenzymes. These GSH S-T represent a family of isoenzymes comprising homo- and hetero- dimers derived from a series of different subunits. As previously mentioned (History and Nomenclature, Section 1.1.), the identification of these isoenzymes has been based on their specific activities with various substrates, M_r of their subunits, isoelectric points, sensitivity to inhibitors, reaction with specific antibodies, peptide maps and amino acid sequences.

However, not all these criteria are adhered to, and this has made the identification and comparison of isoenzymes described in the literature extremely difficult. The problem is compounded by the following:

(i) Enzyme assays are performed in different laboratories under different substrate and temperature conditions. For example, temperatures ranging from 25°C (Habig et al, 1974b) to 37°C (Hayes, 1984) have been used.

(ii) Isoelectric points for isoenzymes are not determined and instead the pH at which an enzyme elutes from a chromatofocusing column is quoted (Hayes, 1984). These values may differ extensively, for example, GSH S-T $Y_{b'}Y_{b'}$ elutes from a chromatofocusing column at a pH of 8.3, whereas it's isoelectric point (determined by isoelectric focusing) is at pH 6.9 (Alin et al, 1985b).

(iii) SDS-polyacrylamide gel electrophoresis is not performed under similar conditions resulting in differences in the M_r values obtained for the various subunits by different laboratories. For example, rat liver GSH S-T subunit Y_c has been reported to have a M_r ranging from 25 000 (Jakoby, 1978) to 30 000 (Ketterer, 1986).

Normal adult rat livers contain six major cationic GSH S-T. These dimeric proteins are composed of subunits termed Y_a, Y_b, $Y_{b'}$ and Y_c. Three of the six isoenzymes (two homodimers and one heterodimer), are composed of subunits Y_a and Y_c while the remaining three isozymes consist of similar combinations of subunits Y_b and $Y_{b'}$. Tables 1.2 to 1.4 list the various properties of the major rat liver GSH S-T described to-date.

The rat hepatic GSH S-T are the products of at least two multigene families (Ding et al, 1985; Li et al, 1986), Y_a-Y_c and Y_b-$Y_{b'}$. The Y_a-Y_c gene family and the Y_b-$Y_{b'}$ gene family have little or no obvious DNA sequence homologies (Ding et al, 1985). Boyer et al (1983) reported the resolution of the Y_a band of GSH S-T Y_aY_a and Y_aY_c into two bands giving the first suggestion of microheterogeneity amongst the subunits of

Table 1.2. **PHYSICAL AND IMMUNOCHEMICAL PROPERTIES OF THE SIX MAJOR RAT LIVER GSH S-TRANSFERASES**

Properties	Isoenzyme					
	Y_aY_a	Y_aY_c	Y_cY_c	Y_bY_b	$Y_bY_{b'}$	$Y_{b'}Y_{b'}$
Apparent subunit M_r	25K	25K + 28K	28K	26.5K	26.5K	26.5K
Isoelectric point	10	9.9	9.8	8.4	8.1	6.9
Precipitin reaction with anti-serum to transferase						
Y_aY_a	+	+	-	-	-	-
Y_aY_c	+	+	+	-	-	-
Y_cY_c	-	+	+	-	-	-
Y_bY_b	-	-	-	+	+	-
$Y_bY_{b'}$	-	-	-	+	+	+
$Y_{b'}Y_{b'}$	-	-	-	-	+	+

(From Alin et al, 1985b)

Table 1.3. **SPECIFIC ACTIVITIES OF THE SIX MAJOR RAT LIVER GSH S-TRANSFERASES TOWARDS SELECTED SUBSTRATES**

Substrate	Isoenzyme specific activity ($\mu mol.min^{-1}.mg^{-1}$)*					
	Y_aY_a	Y_aY_c	Y_cY_c	$Y_{b'}Y_b$	$Y_bY_{b'}$	$Y_{b'}Y_{b'}$
1-Chloro-2,4-dinitrobenzene	50	25	17	58	45	17
1,2-Dichloro-4-nitrobenzene	< 0.04	< 0.04	< 0.04	5.3	3.2	0.18
Bromosulfophthalein	< 0.01	< 0.01	< 0.01	0.94	0.32	0.04
Ethacrynic acid	0.08	0.52	1.24	0.08	0.34	0.62
trans-4-Phenyl-3-buten-2-one	< 0.004	< 0.004	< 0.004	0.05	0.57	1.18
1,2-Epoxy-3-(p-nitrophenoxy)-propane	< 0.1	< 0.1	< 0.1	0.53	0.94	1.37
Cumene hydroperoxide	3.1	5.2	7.9	0.35	0.45	0.72
delta^5-Androstene-3,17-dione	4.2	2.2	0.36	0.02	0.009	0.0002
p-Nitrophenyl acetate	0.79	0.32	0.20	1.01	0.62	0.28

* Specific activities were determined at 30°C.
(From Alin et al, 1985b)

Table 1.4. SENSITITIVIES OF THE SIX MAJOR RAT LIVER GSH S-TRANSFERASES TO SELECTED INHIBITORS

Inhibitor	Isoenzyme I_{50} (μM)*					
	Y_aY_a	Y_aY_c	Y_cY_c	Y_bY_b	$Y_bY_{b'}$	$Y_{b'}Y_{b'}$
Cibacron blue	0.6	4	20	0.25	0.25	0.1
Tributyltin acetate	2	1	0.5	1	1	2
Triethyltin bromide	350	100	3	1	1	70
Triphenyltin chloride	0.5	1	30	0.1	0.1	0.2
Bromosulfophthalein	2	10	200	10	6	0.5
Haematin	0.05	0.2	>2	1	1	1
S-(p-Bromobenzyl)glutathione	50	10	2	25	35	6
Rose bengal	0.5	1	20	1	1	1
Indomethacin	50	100	100	100	100	1

*The I_{50} value is the concentration of inhibitor giving 50% inhibition of the enzyme activity assayed at pH 6.5, 30°C, with 1 mM CDNB and 1 mM GSH as substrates.
(From Alin et al, 1985b)

the GSH S-T. This has subsequently been confirmed for the other subunits as well (Satoh et al, 1985b; Tu and Reddy, 1985; Hayes, 1986a). Tu and Reddy (1985) have demonstrated the possible existence of 10 GSH S-T isoenzymes comprising the Y_a, Y_b, $Y_{b'}$ and Y_c subunits of the GSH S-T. These microheterogeneous subunits are immunologically related to the parent subunit and appear to have very similar substrate specificities and M_r values, but differ by approximately 0.3 of a pI unit within each subunit (Satoh et al, 1985b; Tu and Reddy, 1985).

This multiplicity of isoenzymes with overlapping substrate specificity may be essential to the multiple roles of the GSH S-T in xenobiotic metabolism, drug biotransformation, protection against lipid peroxidation, and cellular ligand transport. Because little data is available on these microheterogenous subunits and because they are so closely related to the "parent" isoenzyme, they will be regarded as being synonymous with the "parent" isoenzyme.

(a) GSH S-Transferase Y_aY_a

This isoenzyme has the high binding affinity which was originally ascribed to ligandin (Litwack et al, 1971). Together, GSH S-T Y_aY_a and Y_aY_c share the physiological role of "ligandin" in reversible binding of non-substrate ligands. The Y_a subunit has a lower affinity for S-hexylglutathione (Alin et al, 1985b) than most other subunits with the exception of transferase E (5-5) and therefore overloading of S-hexylglutathione affinity gels during purification results in decreased yields of GSH S-T Y_aY_a and Y_aY_c. This isoenzyme is effectively induced by xenobiotics such as phenobarbital (Bass et al, 1977a), 3-methylcholanthrene and trans-stilbene oxide (Mannervik, 1985a). The Y_a subunit is associated with high isomerase activity with delta^5-androstene-3,17-dione (Mannervik and Jensson, 1982).

(b) GSH S-Transferase Y_cY_c

The Y_c subunit is associated with high GSH peroxidase activity (Prohaska, 1980; Mannervik and Jensson, 1982). It is therefore probable that GSH S-T Y_cY_c serves a physiological function in protecting cells against lipid peroxidation.

(c) GSH S-Transferase Y_aY_c

This isoenzyme has properties that are generally intermediate between those of the two homodimers Y_aY_a and Y_aY_c (Mannervik and Jensson, 1982; Alin et al, 1985b).

(d) GSH S-Transferase Y_bY_b

The Y_b subunit is associated with high GSH S-aryltransferase activity, particularly with the substrate 1,2-dichloro-4-nitrobenzene (Jakoby and Habig, 1980). The enzyme is also associated with high activity with BSP as substrate (Jakoby and Habig, 1980) and is strongly induced by trans-stilbene oxide, phenobarbital and 3-methylcholanthrene (Mannervik, 1985a).

(e) GSH S-Transferase $Y_{b'}Y_{b'}$

This isoenzyme has high activity with 4-hydroxynon-2-enal (Alin et al, 1985a), a substrate which may be produced in vivo as a result of lipid peroxidation. It is historically characterized by its high activity with trans-4-phenyl-3-butene-2-one (Mannervik and Jensson, 1982). Arene oxides also serve as good substrates for this isoenzyme (Glatt et al, 1983).

(f) GSH S-Transferase Y_bY_b'

GSH S-T Y_bY_b' has properties that are generally intermediate between those of the two homodimers Y_bY_b and Y_b'Y_b' (Mannervik and Jensson, 1982; Alin et al, 1985b).

(g) GSH S-Transferase 5-5(E)

This enzyme is not bound to the S-hexylglutathione affinity matrix commonly used for the purification of the rat GSH S-T (Mannervik, 1985a) and consequently has not been very well characterized. It has high enzymic activity with 1,2-epoxy-3-(p-nitrophenoxy)propane and iodomethane as substrates (Habig et al, 1974b).

(h) GSH S-Transferase Y_tY_t

GSH S-T Y_tY_t is an acidic protein and is the major GSH S-T expressed in the rat testis (Boyer and Kenney, 1985). The Y_t subunit (M_r 26 000) (referred to by Hayes (1984) as Y_n) also exists in two minor heterodimeric forms with a subunit that has an electrophoretic mobility identical to that of the Y_b/Y_b' subunit(s) (Boyer and Kenney, 1985). These two isoenzymes also appear to be acidic proteins (Boyer and Kenney, 1985). Hayes (1984), after devising a purification scheme using hybridized rat testis and liver GSH S-T, reported these two heterodimers to be present in rat liver but others have been unable to identify the Y_t(or Y_n) subunit in normal rat liver (Mannervik and Jensson, 1982; Reddy et al, 1984; Boyer and Kenney, 1985; Tu and Reddy, 1985). GSH S-T Y_tY_t is characterized by its high specific activity with CDNB as electrophilic substrate (Boyer and Kenney, 1985). It is not induced by xenobiotics such as trans-stilbene oxide, phenobarbital or 3-methylcholanthrene (Mannervik, 1985a).

(i) GSH S-Transferase Y_pY_p

This acidic isoenzyme (pI 6.8) was first isolated from rat placenta by Sato et al (Sato et al, 1984; Kitahara et al, 1984) and subsequently from primary rat hepatomas (Meyer et al, 1985) and rat kidney (Guthenberg et al, 1985). Subunit Y_p migrates faster (M_r 24 000) than the Y_a subunit on sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and the isoenzyme serves as a marker for preneoplastic cells in rat chemical hepatocarcinogenesis (Satoh et al, 1985a). The main distinguishing characteristics of the enzyme are (i) GSH peroxidase activity towards fatty acid hydroperoxide and (ii) inactivity towards the model substrates cumene hydroperoxide and t-butyl hydroperoxide (Meyer et al, 1985).

(j) GSH S-Transferase 8-8

GSH S-T 8-8 is one of the less abundant cytosolic proteins accounting for approximately 1% of the total activity with CDNB as substrate (Jensson et al, 1986). It is an acidic isoenzyme (pI 6.0) comprising a subunit (M_r 24 500) which migrates faster than the Y_a subunit on SDS-polyacrylamide gel electrophoresis. The enzyme has high catalytic activity with 4-hydroxyalk-2-enals and ethacrynic acid (Guthenberg et al, 1985; Jensson et al, 1986).

(k) GSH S-Transferase Y_kY_k

This isoenzyme described by Hayes (1986a) is also an acidic isoenzyme which appears similar to GSH S-T 8-8 in that it has a pI of 6.1, has a similar M_r and has high activity with ethacrynic acid as electrophilic substrate. However, Hayes (1986a) reports that it has little affinity for a S-hexylglutathione affinity matrix which was used for the purification of GSH S-T 8-8.

(l) GSH S-Transferase $Y_\alpha Y_\alpha$

This is a basic protein which elutes from a CM-cellulose column after GSH S-T Y_cY_c i.e. at a relatively high ionic strength (Reddy et al, 1984; Tu and Reddy, 1985). It migrates faster than the Y_a subunit on SDS-polyacrylamide gel electrophoresis but no information on its pI is available. This isoenzyme is characterized by its very low activity with CDNB and relatively high activity with 1,2-epoxy-3-(p-nitrophenoxy)-propane (Reddy et al, 1984).

(m) GSH S-Transferase 3-?

Alin et al (1985a) using fast protein liquid chromatofocusing, reported the identification of an isoenzyme that is a heterodimer of subunit $3(Y_b)$ and another subunit with a similar M_r to subunits $Y_b/Y_{b'}$. It is not, however, Y_b or $Y_{b'}$. This subunit may be the same as that referred to by Hayes (1986b) as subunit "Yb_3" and which forms a heterodimer with subunit $Y_{b'}$.

1.4.2. GSH S-Transferases : Extrahepatic Organs

In addition to the major isoenzymes of the liver, testes and kidney (already discussed), the isoenzymes of the lung, heart and brain have also been the subject of investigation.

Robertson et al (1985) found that rat lung contained GSH S-T comprising subunits Y_c, Y_b and $Y_{b'}$ which were similar to the rat liver subunits. In additon, there was a major isoenzyme (transferase "pH 8.7") present in rat lung which was also present in the kidney. It has now been identified as GSH S-T Y_pY_p. They found no trace of isoenzymes comprising the Y_a subunit. However, Awasthi et al (Awasthi et al, 1984; Singh and Awasthi, 1986) have reported the presence of Y_a type subunits in the lung, which, contrary to that generally found in other organs, form heterodimers with the Y_b type subunits. Whether this Y_a type subunit is identical to the liver Y_a subunit remains to be established.

Ishikawa et al (1986) have recently shown that the major GSH S-T isoenzyme in the rat heart is identical to GSH S-T $Y_{b'}Y_{b'}$ (GSH S-T X).

Rat brain has been shown to have subunits corresponding to rat liver GSH S-T subunits Y_b and Y_c. In addition two subunits corresponding to Y_t and Y_p also appear to be present (Kubota et al, 1985; Li et al, 1986).

1.4.3. Microsomal Rat GSH S-Transferase

This membrane-bound enzyme is a very basic protein (pI 10.1) (Morgenstern and DePierre, 1983) comprising 3% of the total microsomal protein (Morgenstern et al, 1983). The purified enzyme has relatively low activity with CDNB but can be activated 15-fold by treatment with N-ethylmaleimide (Morgenstern and DePierre, 1983). A low GSH peroxidase activity with cumene hydroperoxide as substrate can similarly be activated 10-fold. The subunit M_r is approximately 14 000, and in contrast to the dimeric cytosolic GSH S-T, the microsomal enzyme is believed to be a trimeric or tetrameric protein (Morgenstern et al, 1982).

1.4.4. GSH S-Transferases : Human

The human GSH S-T may conveniently be divided into three groups based upon their pI values (Mannervik, 1985a). Thus in the human there are at least five basic transferases termed α to ε in addition to a near-neutral transferase μ and at least two acidic transferases ω and ψ. ω appears to be identical with the placental GSH S-T π or the erythrocyte GSH S-T ρ (Soma et al, 1986). Tables 1.5 and 1.6 list the properties for the three groups of human GSH S-T.

At first a trans-species relationship was not immediately apparent, but substrate specificity and observations of immunological cross-reactivity (Satoh et al, 1985a) between anti-rat GSH S-T and human GSH S-T have enabled relationships to be established (Mannervik et al, 1985) between:

Table 1.5. **CHARACTERISTICS OF THE MAJOR TYPES OF HUMAN GSH S-TRANSFERASES**

Property	Isoenzyme Type		
	Basic	Near-neutral	Acidic
Subunit M_r	25K	26K	23K
Isoelectric point	7.5-9	6.6	4.8
Precipitin reaction with antiserum against GSH S-T type			
Basic	+	-	-
Near-neutral	-	+	-
Acidic	-	-	+

(From Mannervik, 1985a)

Table 1.6. SUBSTRATE SPECIFICITIES OF THE HUMAN GSH S-TRANSFERASES

Substrate	Specific activity ($\mu mol.min^{-1}.mg^{-1}$) Transferases		
	Basic	Near-neutral	Acidic
1-Chloro-2,4-dinitrobenzene	64	187	105
1,2-Dichloro-4-nitrobenzene	0.035-0.065	0.032	0.11
Bromosulfophthalein	0.001-0.01	< 0.002	< 0.002
Ethacrynic acid	0.017-0.044	0.081	0.86
trans-4-Phenyl-3-buten-2-one	0.001-0.002	0.36	0.01
1,2-Epoxy-3-(p-nitrophenoxy)-propane	0	0.11	0.37
Styrene-7,8-oxide	0.02	2.6	0.07
Benzo(a)pyrene-4,5-oxide	0.047	0.92	0.13
Cumene hydroperoxide	10.6	0.63	0.03
delta^5-Androstene-3,17-dione	8.0	0.12	0.01
p-Nitrophenylacetate	0.18	0.22	0.19

(From Mannervik, 1985a)

(i) human GSH S-T α to ε and rat GSH S-T subunit Y_a;

(ii) rat GSH S-T subunit Y_c and mouse GSH S-T MI;

(iii) rat GSH S-T subunits Y_b and $Y_{b'}$ and mouse GSH S-T MIII; and

(iv) human GSH S-T π, rat GSH S-T subunit Y_p and mouse GSH S-T MII.

The closeness of these relationships is apparent from a study of N-terminal amino acid sequences of rat GSH S-T Y_pY_p (Suguoka et al, 1985) and human GSH S-T π (Alin et al, 1985c), which show considerable homology.

Recently it has been shown that a novel isoenzyme purified from human skin (Del Boccio et al, 1987a) is almost identical to GSH S-T Y_cY_c. The most notable similarities being demonstrated in immunological cross-reactivity, amino acid compositions and identical N-terminal amino acid sequences. This suggests that the similarities between enzymes isolated from different species are more extensive than previously assumed.

1.5. STRUCTURE OF THE SOLUBLE RAT GSH S-TRANSFERASES

It is now known that subunits Y_a and Y_c have homology in their amino acid sequences determined either by analysis of tryptic digest peptides (Beale et al, 1982) or by analysis of cDNA sequences (Telakowski-Hopkins et al, 1985). Tryptic peptides of subunits Y_b, $Y_{b'}$ and Y_t also show homology of amino acid sequence, which has been confirmed by the N-terminal sequences of subunits Y_b and $Y_{b'}$ (Frey et al, 1983). cDNA sequences corresponding to mRNAs coding for subunits Y_a, Y_c and Y_p have become available relatively recently (Lai et al, 1984; Taylor et al, 1984; Suguoka et al, 1985; Telakowski-Hopkins et al, 1985), and the amino acid sequences for which they encode show close homology between subunits Y_a and Y_c. Very much less homology is seen with subunit Y_p, even so, the same amino acid residue occupies 26% of the equivalent positions in all three subunits and there are small areas of even higher homology. This homology may be related to a common property such as GSH binding.

Li et al (1986) looked at the expression of the GSH S-T in rat brain by protein purification, in vitro translation of rat brain poly(A) RNAs, and RNA blot hybridization with cDNA clones encoding rat liver GSH S-T subunits, Y_a, Y_b and Y_c. They found the Y_c subunit is expressed in the absence of the Y_a subunit. However, whether the set of Y_c genes expressed in rat livers is the same as that expressed in rat brains, was not positively established even though they shared significant sequence homology and immunological cross-reactivity. Nevertheless, their results suggest that the expression of the Y_c and Y_a genes may be more independent of each other than previously realized and that the Y_a and Y_c genes may be two functionally independent gene families.

In summary, the evidence to date suggests that the GSH S-T are the products of a supergene family (Lai and Tu, 1986), with N- and C-terminal amino acid analyses, tryptic peptide analyses and recombinant DNA technology showing variable degrees of sequence homology between GSH S-T subunits from rat liver as well as between GSH S-T subunits from several rat tissues and human liver (Tu et al, 1986).

1.6. GSH S-TRANSFERASES : OCCURENCE IN NATURE

The GSH S-T are extremely widely distributed in nature. GSH S-T activity has been described in bacteria (Summer et al, 1980), filaria (Jaffe and Lambert, 1986), plants (Mozer et al, 1983), many different insects (Clark et al, 1973; Usui et al, 1977; Motoyama and Dauterman, 1978), earthworm (Stenersen et al, 1979), marine crustaceans (Tate and Herf,1978; Bend et al, 1977; Keeran and Lee, 1987), etc. In vertebrates GSH S-T activity has been described in man (Kamisaka et al, 1975a), rat (Booth et al, 1961; Pabst et al, 1973), mouse (Clark et al, 1973), guinea pig (Irwin et al, 1980), monkey (Asaoka et al, 1977), hamster (Smith et al, 1980), chicken (Yeung and Gidari, 1980), cow (Saneto et al, 1980), sheep (Clark et al, 1973), camel (Hunaiti and Abu Khalaf, 1986), rabbit (Juchau and Namkung, 1974), shark (Sugiyama et al, 1981) trout (Nimmo et al, 1979), snake (Stafford et al, 1976), etc. Stenersen et al (1987) have recently shown GSH S-T activity in 71 of 72 animal species/stages representing nine phyla from aquatic and terrestrial animals.

1.7. GSH S-TRANSFERASES : TISSUE DISTRIBUTION

GSH S-T have been shown to be present in all rat and human organs studied, from the steroidogenic tissues to the brain (Kirsch et al, 1975; Bass et al, 1977b; Campbell et al, 1980; Baars et al, 1981; Sherman et al, 1983; Eidne et al, 1984; Singh et al, 1985; Corrigall et al, 1986; Dijkstra et al, 1986; Del Boccio et al, 1986; Del Boccio, 1987b). At the cellular level a major portion of the soluble forms are found in the cytosol with traces present in mitochondria and nucleoplasm (Kraus and Gross, 1979; Kraus, 1980). Membrane-bound forms are found in the mitochondria and endoplasmic reticulum (Morgenstern et al, 1982).

Determining the concentration and tissue distribution of these enzymes has proved difficult due to their multiplicity. Most studies to date have the disadvantage of having been performed with a specific substrate rather than a specific isoenzyme. The overlapping substrate specificity of these enzymes and the existence of new as well as incompletely characterized isoenzymes makes the interpretation of these results difficult. Studies using antibodies to a specific isoenzyme are confounded by the existence of heterodimers and cross-reactivity with new or incompletely characterized isoenzymes bearing antigens shared with the specific isoenzyme. Baars et al (1981) have looked at GSH S-T activity in rat and human tissues using the substrate 1-chloro-2,4-dinitrobenzene. In man the kidney and liver had the highest specific activity while in the rat the highest activity was present in the liver. In man, muscle also had a relatively high specific activity (10-fold higher than the rat). Considering the overall contribution of skeletal muscle to body mass, the major isoenzyme comprising skeletal muscle is probably the most prevalent isoenzyme in man. Many interesting observations relating to tissue distribution and function of the enzymes can be made. Of importance is the presence of GSH S-T in those organs or tissues which are the entry or exit sites of xenobiotics such as the gastrointestinal tract, lungs, skin, and in particular the liver and kidneys. In these organs the GSH S-T may account for approximately 3-10% of the soluble proteins (Jakoby et al, 1976; Jakoby and Habig, 1980). A further interesting point is the high specific activity of these enzymes in the testis of man and rat. If the enzymes were functioning in detoxification, then high levels of transferases in the spermatogenic tissue would be expected to protect genetic material from xenobiotic damage during its production. Probably of major significance is the considerable inter-organ and inter-individual variation in the expression of the various isoenzymes (Sherman et al, 1983; Corrigall et al, 1986). These variations may explain individual and organ susceptibility to the toxic effects of xenobiotics.

1.8. ONTOGENY OF THE GSH S-TRANSFERASES

1.8.1. Rat Studies

Little is currently known about the ontogeny of the GSH S-T. Most studies to date have the disadvantage of having been performed with a specific substrate rather than a specific isoenzyme.

In the early 1960's Combes and Stakelum (1962) found the conjugation of BSP to be significantly reduced in the livers of fetal and newborn rats. Grodsky et al (1970) found the binding activity of fetal rat liver (2 days prepartum) for bilirubin and BSP to be 25-35% of that in the adult with adult levels not being attained before 30 days postpartum. Grodsky et al and Levi et al (1970) suggested that the immaturity of a carrier system for bilirubin in the newborn rat may provide an explanation for the physiological jaundice and hyperbilirubinemia often observed in newborn humans.

Klaasen (1975) found that the amount of "ligandin" (determined by BSP-binding) in the livers of five-day-old rats was approximately 10% of that found in the adult. This amount increased slowly during the first 15 days of life and then rose rapidly to peak at 35 days of age after which it reached a plateau. The microsomal enzyme inducer, preg-nelone-16alpha-carbonitrile, administered to rats six to nine days after birth more than doubled the amount of ligandin present in the livers on day 10.

Mukhtar and Bresnick (1976) looked at the specific activity of the GSH S-T towards epoxide substrates in both rat liver and lung during development from the fetal to the adult stage. They found that enzyme activity in fetal liver or lung at 18 days of gestation (gestation time is 21 to 22 days) was about 20% of that observed in the adult, rising rapidly to the adult value by 21 days of age.

Hales and Niems (1976) looked at the postnatal development in male rats of hepatic GSH S-T B (Y_aY_c) using a combination of substrate specificity and immunotitration with antibodies to GSH S-T Y_aY_c. They found that the concentration of GSH S-T Y_aY_c in one-day-old rats was about 20% of that in adult animals reaching adult levels four to five weeks after birth. The proportion of GSH S-T Y_aY_c relative to the other GSH S-T was high during the first week after birth. At this age 67.5% of the activity towards CDNB was immunoprecipitable by anti-Y_aY_c antiserum compared with about 50% in adults. Between the second and fifth weeks after birth, the fraction of GSH S-T Y_aY_c increased in parallel fashion with the other transferases. They found that phenobarbital induced GSH S-T activity towards CDNB and DCNB in both rat pups and adults, the percentage increase remaining constant during maturation i.e. a function of the basal value.

Bhargava et al (1983) found that eight-week-old rats had a twofold higher hepatic ligandin concentration than ten-day-old animals determined immunologically and by steroid isomerase and GSH S-T assays, thus confirming the findings of Hales and Niems (1976). The mRNA content for each "ligandin" subunit (Y_a and Y_c) was twofold higher in older animals determined by cell-free in vitro translation followed by immunoprecipitation and dot hybridization using a ligandin cDNA probe.

Eidne et al (1984) looked at the postnatal development of GSH S-T Y_aY_c in the steroidogenic tissues of the rat using a radioimmunoassay.

They found that the levels in testis, ovary and adrenal tissue were relatively high (in testis and adrenals they reached adult levels) on the fourth day after birth. These levels decreased by day 9 and increased during puberty to reach adult levels. In contrast, they found that levels in the liver and kidney were low at birth and rose progressively to adult levels. Phenobarbital was unable to induce the levels of GSH S-T Y_aY_c in the steroidogenic tissues.

The ontogeny of the GSH S-T in rat brain received very little attention until recently when Senjo et al (1986) reported their findings on GSH S-T activity in fetal and postnatal rat brains towards the substrate CDNB. They looked at fetal development from day 16 of gestation to day 56 postnatally. They found that GSH S-T activity was first detectable in the fetus at day 18 of gestation and that postnatally, from day 5, GSH S-T activity increased rapidly.

Di Ilio et al (1986) have recently looked at the activities of the GSH S-T in fetal rat livers at days 16 and 20 of gestation towards three substrates, namely, CDNB, DCNB and 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNPP). They found that at day 16 of gestation the activities towards CDNB, DCNB and EPNPP were 4, 3 and 105% of their respective adult values. At day 20 these values had changed to 9, 26 and 73% respectively. Selenium-independent GSH peroxidase activity appeared to remain constant during fetal development. This confirms earlier findings including those of Baars et al (1980) that the development of various hepatic GSH S-T is not homogenous.

1.8.2. Other Animals

James et al (1977) looked at the perinatal development of the GSH S-T using styrene oxide and DCNB as substrates in the liver, lungs, kidneys and small intestine of fetal and neonatal guinea pigs and rabbits. They found that during gestation the activity in fetal rabbit liver (third trimester onwards) towards the epoxide substrate was only 5-10% of the adult value, and it increased sharply between 1 and 6 days after birth to 50% of the adult value. Little change occurred between 6 and 28 days of age reaching adult levels at 70 days of age. In extrahepatic tissues there was a fall in activity around birth but the rate of increase in activity postnatally was different in the three extrahepatic tissues. Thus six days after birth, activities in the intestine and lung were only about 40% of adult values, whereas kidney specific activity was within the adult range. In guinea pig liver the specific activity increased rapidly after birth and was within the adult range by 3 days of age. The activity in lungs was comparable to adult values at all ages from gestational day 54 (gestation time is usually 68 days) onward and were higher than adult values at 67 days of gestation and 1 day after birth. Activity in renal cytosol fractions rose gradually from 15% of adult levels at 38 days of gestation to reach adult activity by 25 days postnatally. In contrast, the activity in the intestine increased gradually compared to the other organs. Activities with DCNB as substrate showed similar developmental patterns in the organs of both rabbits and guinea pigs.

Similar postnatal developmental patterns to those obtained in the rat liver have been obtained by others for the mouse liver

(Hatayama et al, 1986; Harman and Henry, 1987).

1.8.3. Human Studies

In 1974 Juchau and Namkung published their findings on the biotransformation of naphthalene-1,2-oxide by the GSH S-T in fetal and placental tissues of humans and monkeys. They found that in particular monkey fetal liver and to a lesser degree human fetal liver had higher specific activities with this substrate than adult rat and rabbit livers. Unfortunately they did not determine activities in fetal rat and rabbit livers. Of interest was the inter-organ and inter-species differences in GSH S-T activity to this substrate.

Studies on the human GSH S-T did not advance further until the 1980's when Mukhtar et al (1981) looked at the GSH S-T activity in human fetal and adult tissues at autopsy using CDNB as electrophilic substrate. They found that liver, lung, muscle kidney, heart, adrenal glands, pancreas and stomach of fetal origin had higher specific activities than those of the adults studied, including wide interindividual variations in tissue enzyme activities. In the fetal liver, activity was not detectable before 16 weeks of gestation in contrast to the small intestine where activity was detectable at 12 weeks.

Polidoro et al (1982) found CDNB activity in fetal livers obtained from legal first trimester therapeutic abortion. However, they found no detectable activity with DCNB, EPNPP or p-nitrobenzylchloride as substrates. Isoelectric focusing showed two groups of enzymes to be expressed in fetal livers, one basic and the other acidic.

Fryer et al (1986) have found that human fetal lung has high levels of acidic isoenzyme which decrease markedly after 15 weeks of gestation, with levels at birth only 20% of that in the first trimester. This acidic 'enzyme' may in fact comprise more than one enzyme, one or more of which disappear with increasing fetal development.

Guthenberg et al (Warholm et al, 1981; Guthenberg et al, 1986) have looked at the expression of the GSH S-T in the human fetal liver and found two distinct forms of GSH S-T, one acidic and the other basic, in the liver of a fetus of 25 weeks gestational age. This is contrary to that found in the adult liver which contains only relatively small amounts of the acidic form. They also report an additional acidic transferase in the livers of some fetuses of 19-25 weeks gestational age, the reason for which has not been elucidated. The acidic and basic forms from fetal livers showed extensive similarities with the adult acidic and basic forms, with the exception that both fetal enzymes showed considerably greater catalytic activity with styrene 7,8-epoxide than the corresponding adult forms. The fetal basic form also showed a notable difference from the adult basic forms in its sensitivity to the inhibitor tributyltin acetate. Guthenberg et al have speculated that the human fetal GSH S-T isoenzymes may be separate but very similar forms of enzyme to those present in adult human liver.

Other studies on the isoenzyme expression in extrahepatic tissues (Pacifici et al, 1986) using isoelectric focusing have shown that human fetal lung, kidney, brain and intestine contain only one major form of GSH S-T which focuses at a pH of approximately 4.8. This isoenzyme pattern is similar to that found in human placenta, and adult brain, lung and erythrocytes. In contrast, fetal adrenal gland exhibited a major basic isoenzyme in addition to an acidic isoenzyme i.e. identical to that found in fetal liver, whereas the human adult adrenal gland like the liver posseses mainly basic isoenzymes with small amounts of acidic and in some individuals near-neutral isoenzyme.

Recently Faulder et al (1987) have looked at the fetal and perinatal ontogeny of basic, near-neutral and acidic GSH S-T isoenzymes in human liver, adrenal, kidney and spleen using chromatofocusing and ion-exchange chromatography. They found that :

(i) basic isoenzymes were identified in all liver and adrenal cytosols obtained after 10 weeks gestation, these forms not being detected in kidney until 10 weeks post-natally and in spleen until about 40 weeks post-natally.

(ii) expression of the near-neutral isoenzymes was usually weak and they were not generally expressed in liver until 30 weeks gestation. No developmental patterns in their expression could be identified in adrenal, kidney and spleen.

(iii) the acidic isoenzymes were usually strongly expressed in adrenal, kidney and spleen although there was a decline in the level of expression in kidney after birth.

In summary, the data from both animal and human studies suggest that fetal forms of the GSH S-T in specific organs such as the liver, lungs and adrenal gland may well exist.

1.9. SEX DIFFERENCES IN THE EXPRESSION OF THE GSH S-TRANSFERASES

Sex differences in the ability of the mammalian liver to metabolize xenobiotics appear with the onset of puberty and are maintained throughout life (Dauterman, 1980).

Studies in adult rats have shown sex differences in specific activities to different electrophilic substrates (Kaplowitz et al, 1975; Clifton et al, 1975; Fujita et al, 1985). Recent studies using CDNB and DCNB as electrophilic substrates (Igarashi et al, 1987) have shown that following birth there is an increase in GSH S-T activity in both males and females with no sex differences being evident until approximately five weeks of age. Activity in both sexes continues to rise up to 7 weeks of age and is then followed by a very gradual decline in activity in both males and females. In females at 11 weeks of age there is a rebound increase in activity with CDNB as substrate but by 20 weeks of age this has not reached the values obtainable at 7 weeks of age and is still lower than the activity in males. Immunotitration (Hales and Niems, 1976; Igarashi et al, 1985) and subunit densitometric measurements following sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Igarashi et al, 1987) suggest that these sex differences are related to differences in subunit composition of the GSH S-T in males and females (see Fig. 1.3). These differences like those occurring with the microsomal enzymes are probably due to the influence of the sex hormones. This is substantiated by the finding that hypophysectomy eliminates sex differences in the hepatic concentration of GSH S-T B (Hales and Niems, 1976) and that the ontogeny of ligandin in the testis, ovary and adrenal

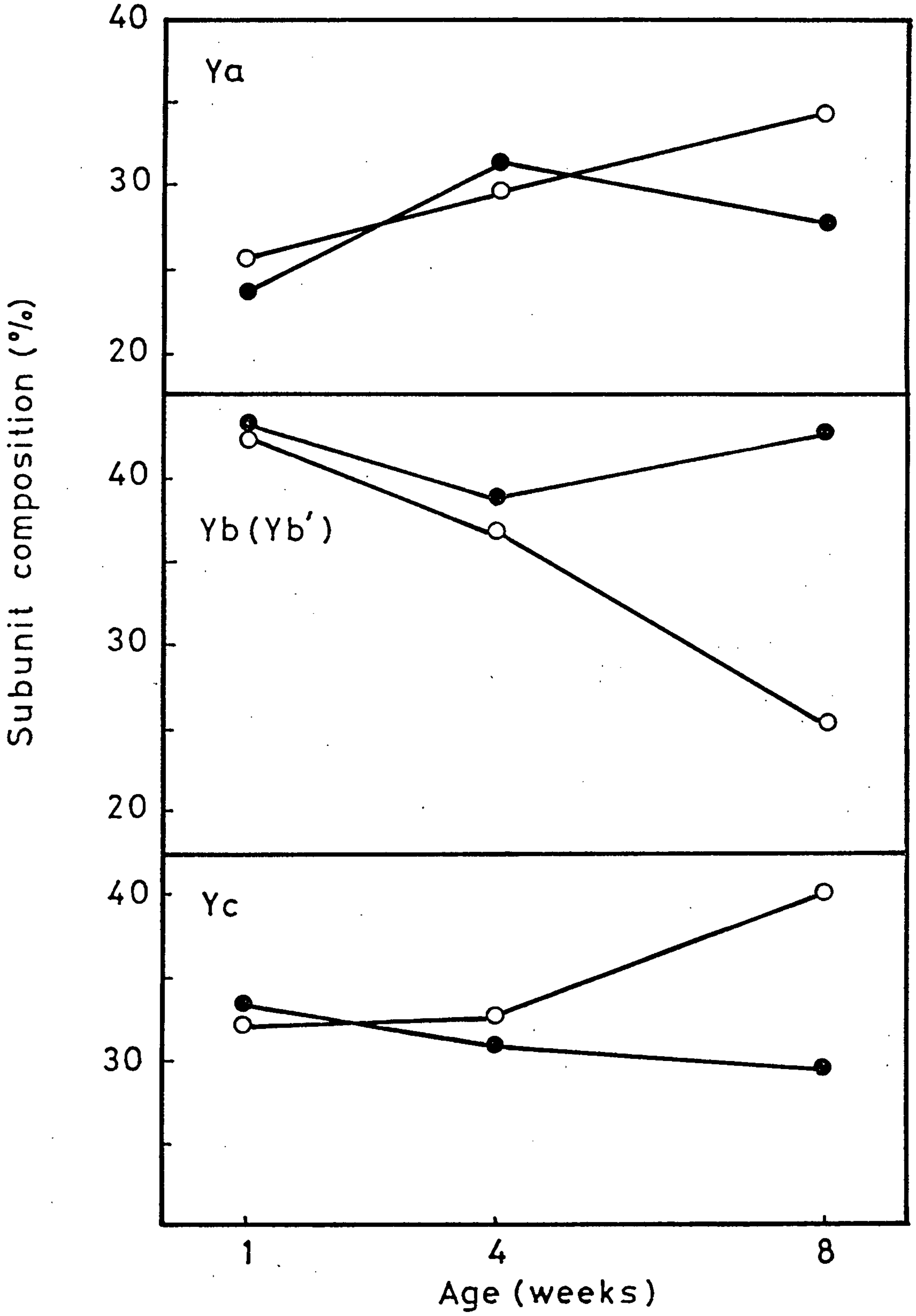


Fig. 1.3. Change in subunit composition of male (●) and female (○) rat liver cytosol with age (From Igarashi et al, 1987).

tissue parallels changes in the circulating levels of testosterone and progesterone. Ligandin levels are relatively high soon after birth, they decrease by day 9 and increase rapidly during puberty to reach adult levels (Eidne et al, 1984).

1.10. INDUCTION OF THE GSH S-TRANSFERASES

The GSH S-T may be induced by two classes of xenobiotics, namely, phenobarbital and polycyclic hydrocarbons (3-methyl-cholanthrene and 3,4-benzopyrene); as well as certain organochlorine compounds (Chasseaud, 1979). Both phenobarbital and the polycyclic hydrocarbons induce liver GSH S-T activity to a range of substrates (Kaplowitz et al, 1975). Bass et al (1977a) found that phenobarbital treatment in adult rats resulted in significant increases in hepatic, renal and small intestinal mucosa ligandin concentrations, with no change in the testis. The increase in V_{max} in response to drug treatment is unaccompanied by an effect on the K_m value indicating that no alteration of substrate affinity for enzymes occurs as a result of the inducing xenobiotics (Kaplowitz et al, 1975). Jakoby and Habig (1980) report that following phenobarbital induction, GSH S-T B may account for up to 13% of the total extractable protein from rat liver and the transferases as a whole may account for almost 20% of the extractable protein. The fact that both the GSH S-T and the microsomal drug-metabolizing enzymes are induced by the same xenobiotics indicates an important cellular relationship between the two groups of enzymes, strengthening the hypothesis that an important role of the GSH S-T is the detoxification of electrophilic products produced by phase I metabolic enzymes such as the microsomal enzymes.

Genotoxic xenobiotics share the common denominator of being either inherently electrophilic or being metabolically activated to electrophilic species (Miller and Miller, 1981). Increased reaction rates of active carcinogens, mutagens and teratogens through enhancement of the GSH S-T conjugation system, will decrease the probability that they will react with DNA and other critical nucleophiles. An increasing number of compounds have been found to inhibit the neoplastic effects of chemical carcinogens when administered before and/or simultaneously with the carcinogen (Wattenberg, 1978). Benson et al (1978; 1979) found that 2(3)-tert-butyl-4-hydroxyanisole which is very effective in inhibiting the neoplastic effects of a wide range of chemical carcinogens when administered in the diet, produced a marked increase in GSH S-T activity. The effect was most pronounced in the liver where GSH S-T activity was enhanced eightfold with small increases occurring in other tissues.

A considerable number of dietary constituents have been found to protect against the occurence of neoplasia (Wattenberg, 1983). Many of these constituents have been found to produce marked differences in GSH S-T levels. Sparnins et al (1982) found that mice fed an unrefined diet had higher GSH S-T activities in the liver and small bowel mucosa than mice fed a semi-purified diet. The addition of dried and powdered green coffee beans, cabbage, brussel sprouts (both the cabbage and brussel sprouts were grown without the use of chemical pesticides) or tea leaves to the semi-purified diet significantly increased GSH S-T in both organs. Investigation has shown that the consumption by mice of a diet containing green coffee beans enhanced the GSH S-T activity of the liver 6-fold and of the small bowel mucosa 7-fold (Wattenberg, 1983). The inclusion of dried coffee beans in the diet of female rats prior to the administration of the mammary tumor inducer 7,12-dimethylbenz(a)anthracene significantly reduced the incidence of neoplasia

(Wattenberg, 1983). More recently Ansher et al (1986) have found that the oral administration of the dithiolthione, oltipraz, (contained in cabbage and other cruciferous vegetables) increased the activities of the GSH S-T to two substrates in the liver, lung, upper jejunal mucosa and kidney of mice. Oltipraz inhibits carcinogenesis and reduces the effects of gamma radiation and the effects of some hepatotoxic agents in mice.

Another study looked at the effect of the induction of the GSH S-T in benzo(a)pyrene-induced mouse forestomach neoplasia. The data indicated that enhancement of GSH S-T activity by 75% or more reduced the incidence of neoplasia significantly (Sparnins and Wattenberg, 1981). With some GSH S-T inducers, formation of these tumors was inhibited by up to 80%.

The GSH S-T in plants can also be induced. Mozer et al (1983) found the GSH S-T in corn were induced by chemicals such as antidotes to certain herbicides, and that induction resulted in the expression of an isoenzyme undetectable in untreated corn. Another form of isoenzyme induction occurs in primary hepatoma of the rat, where the rat placental isoenzyme (Satoh et al, 1985a) is induced. A similar, immunologically related, isoenzyme is overexpressed in human breast cancer cells which exhibit resistance to a wide range of anti-cancer drugs (Batist et al, 1986). The hepatoma isoenzyme normally only attains significant quantities in the kidney and pancreas, but in hyperplastic nodule-bearing livers it attains levels 10-fold higher than in normal liver. The placental GSH S-T in primary hepatoma differs from other transferases in that it is not induced by administration of drugs and carcinogens prior to appearance of preneoplastic foci and hyperplastic nodules and serves as a marker protein for preneoplasia in rat chemical hepatocarcinogenesis (Sato et al, 1984; Satoh et al, 1985a).

1.11. THE GSH S-TRANSFERASES AND LIPID PEROXIDATION

1.11.1. Glutathione Peroxidases

Glutathione peroxidases catalyze the reduction of hydroperoxides (ROOH) by GSH as follows:

$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$$

R may be an aliphatic or aromatic organic group, or hydrogen (Mannervik, 1985b). The products are H_2O, an alcohol (ROH)(or a second H_2O) and glutathione disulfide (GSSG, i.e., oxidized GSH). Cellular regeneration of GSH from GSSG is effected enzymatically by glutathione reductase in a $NADPH_2$-dependent reaction (see Fig. 1.4).

Two major types of GSH peroxidase have been found. One contains selenium in the form of covalently bound selenocysteine in its active site. The second type of GSH peroxidase consists of proteins that do not depend on selenium for catalysis and are characterized by their inability to reduce H_2O_2.

1.11.1.1. Selenium-Dependent Glutathione Peroxidase

Glutathione peroxidase (glutathione: hydrogen peroxide oxidoreductase, EC 1.11.1.9)(GSH peroxidase) was first detected by Mills (1957) in erythrocyte lysates where it was found to protect

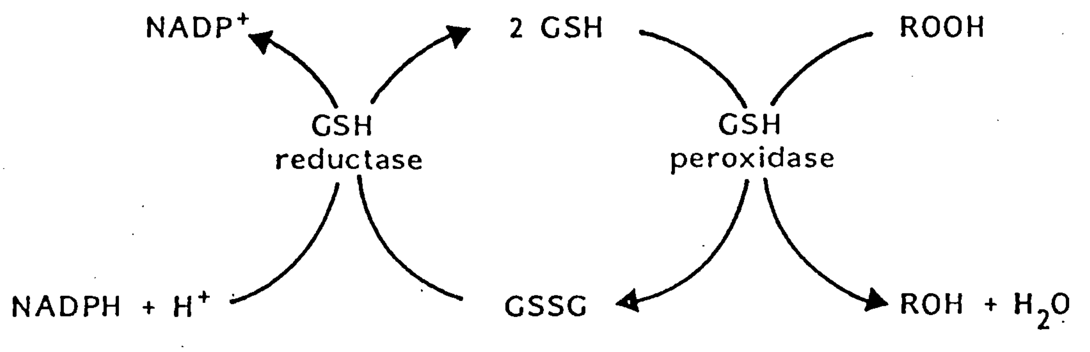


Fig. 1.4. Outline of GSH peroxidase and GSH reductase action (From Bhargava and Arias, 1981).

haemoglobin from oxidative breakdown. In 1959 Mills isolated GSH peroxidase from bovine erythrocytes and showed that it was distinct from catalase (considered the primary scavenger of intracellular H_2O_2) and haemoglobin. In 1963 Cohen and Hochstein published a paper in which they presented evidence to show that in erythrocytes GSH peroxidase rather than catalase served the primary role in H_2O_2 detoxification. However, Oshino and Chance (1977) using the isolated perfused rat liver as a model found that within the peroxisome it was in fact catalase which performed this primary function. GSH peroxidase was found to play a more important role in other subcellular fractions such as mitochondria and cytosol. GSH peroxidase has also been shown to protect isolated hepatocytes from H_2O_2 produced as a result of microsomal oxidative demethylation where the formate that is produced inhibits catalase and may significantly reduce its contribution to H_2O_2 removal (Jones et al, 1978).

This selenium-dependent enzyme is able to reduce both organic hydroperoxides and H_2O_2. The enzyme from bovine erythrocytes is tetrameric comprising four identical spherical monomers each containing a selenium atom (Ladenstein et al, 1979). Cyanide inhibits the purified enzyme resulting in loss of enzyme activity and release of selenium from the enzyme (Prohaska et al, 1977). However, under physiological conditions the enzyme appears resistant to cyanide inhibition. Cadmium and other heavy metals have also been shown to have an inhibitory effect on GSH peroxidase (Splittgerber and Tappel, 1979).

Immunohistological studies suggest that rat liver GSH peroxidase is located in the cytoplasm of hepatocytes, with more intense staining in the periportal areas than the central zone of the lobule (Yoshimura et al, 1980).

1.11.1.2. Selenium-Independent Glutathione Peroxidases

In 1976 Lawrence and Burk reported that the GSH peroxidase activity in the liver supernatant obtained from rats that had been fed a selenium-deficient diet for 2 weeks was 8% of control values when assayed with H_2O_2 as substrate but 42% of control values when assayed with cumene hydroperoxide. This provided the first evidence for the existence of a second GSH peroxidase enzyme. They found that the fractionation of liver cytosol on Sephadex G-150 columns provided two peaks of activity. The first (which was absent from selenium-deficient rat liver supernatants) could be detected with H_2O_2, while the second peak could only be detected with cumene hydroperoxide. This peak was found to persist in severe selenium deficiency.

This GSH peroxidase differed from the selenium-dependent form in that :

(i) it did not incorporate ^{75}Se in vivo suggesting it was not a selenoenzyme,

(ii) it showed zero-order dependence on GSH concentration (Prohaska and Ganther, 1976), and

(iii) it was not inhibited by cyanide.

The discovery that the selenium-independent GSH peroxidase activity was accounted for by the GSH S-T was made by Prohaska and Ganther (1977). They had hypothesized that the selenium-independent

GSH peroxidase activity might be accounted for by the ability of the GSH S-T to bind organic hydroperoxides and GSH in close proximity, thereby promoting nucleophilic attack by GSH on the electrophilic oxygen. They were able to co-purify selenium-independent GSH peroxidase activity and GSH S-T activity, thus proving that the GSH S-T were responsible for selenium-independent GSH peroxidase activity.

The results of Prohaska (1980) suggest that the GSH S-T catalyze nucleophilic attack by GS^- on hydroperoxides resulting in a reactive intermediate, probably the sulfenic acid of GSH, GSOH.

$$\mathrm{GSH} + \mathrm{ROOH} \leftrightharpoons \mathrm{GSOH} + \mathrm{ROH}$$

This sulfenic acid then reacts non-enzymatically with GSH to produce GSSG.

$$\mathrm{GSOH} + \mathrm{GSH} \leftrightharpoons \mathrm{GSSG} + \mathrm{H_2O}$$

1.11.2. Occurence

There is wide inter-species variation in the expression of the two forms of enzyme. The selenium-independent enzyme is the predominant form in the livers of humans, guinea pigs, sheep and chickens, while the selenoenzyme predominates in rats and hamsters (Lawrence and Burk, 1978). The ratio between the two forms may not only vary between species, but also from tissue to tissue in the same species. For example, in the rat testis the selenium-independent GSH peroxidase accounts for 91% of the total peroxidase activity (Lawrence and Burk, 1978). It follows that in selenium deficiency the relative importance of the peroxidase activity of the GSH S-T increases.

1.11.3. Subcellular Localization

In rat hepatocytes selenium-dependent GSH peroxidase is absent in peroxisomes and is localized primarily in the cytosol and in the matrix of mitochondria (Flohe and Schlegel, 1971). Mitochondrial extracts from rats fed a selenium-deficient diet have very little GSH peroxidase activity (Sies and Moss, 1978), suggesting that the selenium-independent peroxidases are not important in these organelles. A major portion of the soluble forms of the GSH S-T are found in the cytosol, with traces present in mitochondria and nucleoplasm (Kraus and Gross, 1979; Kraus, 1980). Membrane-bound forms are found in the mitochondria and endoplasmic reticulum (Morgenstern et al, 1982). The microsomal GSH S-T, which is distinct from the cytosolic isoenzymes, also has GSH peroxidase activity with cumene hydroperoxide as substrate (Morgenstern and DePierre, 1983). The microsomes also contain the major 'cytosolic' isoenzymes and judging from quantitative determinations of relative amounts as well as specific activities with cumene hydroperoxide as substrate, it would seem that the peroxidase activity of the 'cytosolic' isoenzymes in the microsomes are quantitatively as important as the so called microsomal enzyme (Mannervik, 1985b).

1.11.4. Lipid Peroxidation

The generation of reactive oxygen species occurs during normal cellular metabolism, the production of which may be influenced by xenobiotics either during oxidative metabolism at the endoplasmic reticulum (e.g. carcinogens and paracetamol), through one electron redox-cycling (e.g. manadione and adriamycin), or by photoactivation (e.g. porphyrins and psoralens) (Ames, 1983). These oxygen species may initiate lipid peroxidation by the abstraction of a hydrogen atom from an unsaturated phospholipid. Fig. 1.5 shows the process of lipid peroxidation (Demopoulos et al, 1980)

1. Part of a fatty acid chain with two unsaturated bonds (can also be one unsaturated bond). X· is a free radical (a substance with a lone electron) and most often represents molecular O_2 (a diradical) or the hydroxyl radical (·OH).

2a. A radical centre is located on the carbon that is adjacent to carbons with double bonds, and is now referred to as an alkyl radical; the original free radical (X·) has abstracted a hydrogen to initiate radical damage to this lipid and is now a non-reactive product (XH).

2b. Several configurational changes occur almost immediately; there

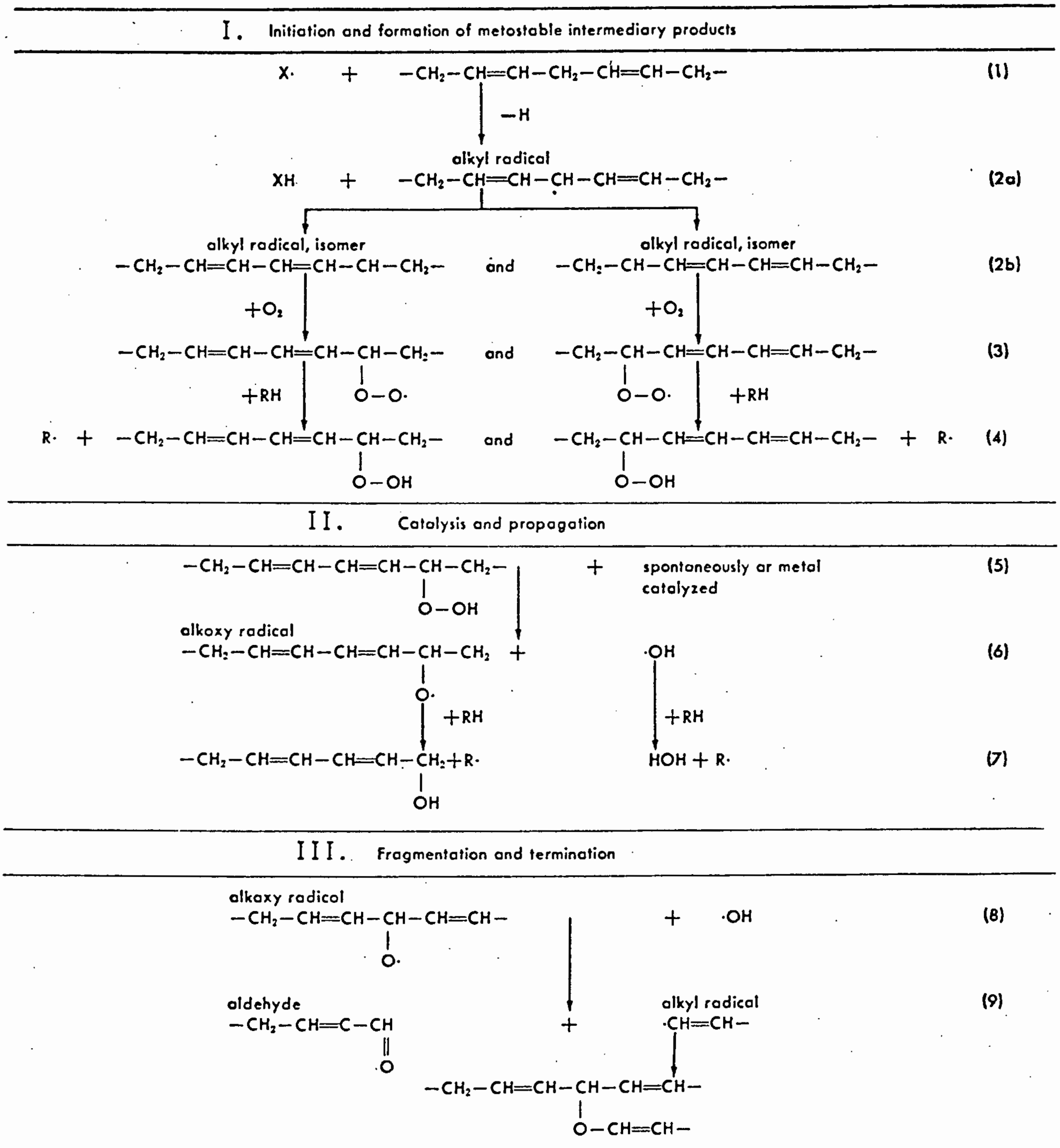


Fig. 1.5. Three basic phases of lipid peroxidation (From Demopoulos et al, 1980).

are massive electron shifts which (i) result in the lone electron being shifted to other carbon atoms, (ii) cause the double bonds to move closer together and thus form conjugated unsaturations, which can be detected spectrophotometrically as a sign of early radical damage, (iii) some double bonds adopt the trans configuration, instead of the cis configuration characteristic of fatty acids.

3. Oxygen adds by free radical reactions to form peroxy radicals (ROO·).

4. The peroxy radicals have abstracted hydrogen from nearby molecules (RH, which may be other unsaturated fatty acids, proteins, antioxidants, or nucleic acids) and have become metastable (transiently stable) lipid hydroperoxides (ROOH).

5-6. A hydroperoxide can break up spontaneously to form hydroxy radicals (·OH) and oxygen-centered radicals on the lipid termed alkoxy radicals (RO·). As a result of metal catalysis, alkoxy and peroxy radicals with hydroxyl or hydrogen ions are produced.

7. Owing to the abstraction of hydrogens from adjacent molecules, RH, the alkoxy and hydroxyl radicals are terminated, but additional radicals form in the molecules that had their hydrogen abstracted (2R·).

8. Alkoxy radicals can be further oxidized and fragmented as a result of continued radical attacks on other carbons that are adjacent to the double bonds. In this way bis-hydroperoxides (i.e. two -OOH groups on a single fatty acid) may be formed and the fatty acid then fragments to form aldehydes and alkyl radical fragments.

9. Alkyl radicals can react with other surrounding radicals, e.g. other alkoxy radicals, and thus the process is terminated as a result of the formation of an oxygen-linked bridge between the molecules, or C-C bonds may be formed with another alkyl radical.

Initiation of lipid peroxidation may thus start a chain reaction which, if uncontrolled by defence mechanisms, may lead to extensive damage of the surrounding molecules (Feher et al, 1987). Autooxidation of lipids eventually leads to the formation of new C-C bonds and since lipids are membrane constituents, cross-linking may cause severe damage to membranes (Fig. 1.6).

In addition, a number of major proteins (e.g. NaK ATPase) derive their active configurational shape as a result of intimate interactions between the hydrophobic portions of the proteins and the fatty acid tails of specific membrane phospholipids (Demopoulos et al, 1980). Because of the susceptibility of the phospholipid fatty acids to radical damage, such proteins can be viewed as having a liability in depending on phospholipid integrity.

Autooxidation is accelerated by the presence of transition metals (copper, iron) enhancing the degradation of metastable hydroperoxides

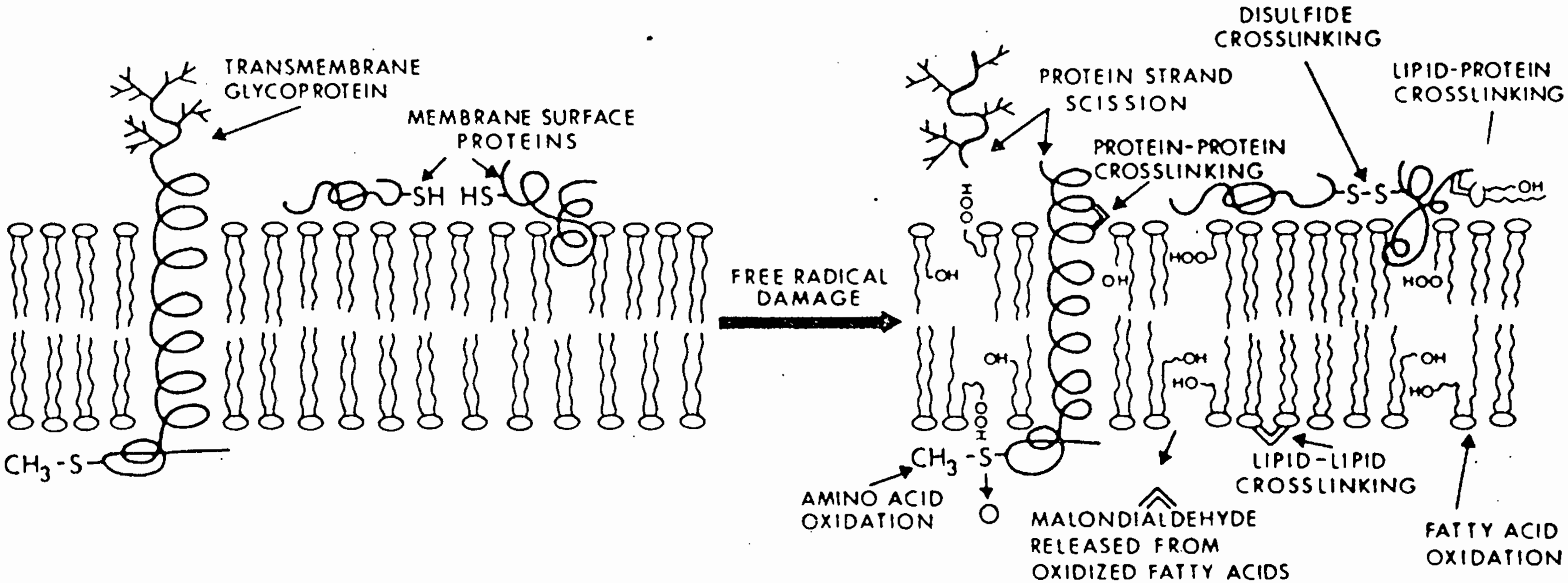


Fig. 1.6. Free radical damage to membranes. Free radicals can affect lipids by initiating peroxidation, which leads to short chain fatty acyl derivatives and the by-product malondialdehyde. A variety of cross-linking reactions can be mediated by malondialdehyde reactions. Free radicals can also catalyze amino acid oxidation, protein-protein cross-linking, and protein strand scission (From Freeman and Crapo, 1982).

(Fig. 1.5, Eqs. 5-6). In the presence of O_2, lipids which are readily oxidized promote the metal-catalyzed oxidation of more inert cellular components such as nucleic acids or proteins (Fig. 1.5, Eqs. 7-9). Although autooxidation is slow under physiological conditions, under pathological conditions it is accelerated, becomes extensive and may damage all basic molecules (Demopoulos et al, 1980; Slater, 1984). The toxic metastable products formed during lipid peroxidation including the mutagen, malondialdehyde, and others such as 2-alkenals and 4-hydroxyl-2-alkenals, may migrate reaching distant parts of the cell, or other cells and tissues causing damage at these distant loci. Lipid peroxidation may thus also damage cells or tissues which are not directly exposed to peroxidative damage (Petkau, 1980).

1.11.5. Detoxification of Lipid Hydroperoxides

Several mechanisms exist to inhibit lipid peroxidation (Demopoulos et al, 1980). One mechanism involves the removal and detoxification of fatty acyl hydroperoxide. This depends on the consecutive action of phospholipase A_2, and the reduction of released free fatty acid hydroperoxide to fatty acid alcohol, by selenium-dependent or selenium-independent GSH peroxidase activity (Tan et al, 1984). None of these enzymes can reduce esterified fatty acid hydroperoxides (Grossmann and Wendel, 1983; Sevanian et al, 1983), and consequently inhibition of lipid peroxidation by GSH peroxidases depends on the co-operative action of phospholipase A_2 (i.e. the GSH peroxidases can only act on free fatty acyl hydroperoxides, the release of which is dependent on phospholipase A_2). Thus inhibition of lipid peroxidation by GSH peroxidases can be prevented by phospholipase A_2 inhibitors (e.g. p-bromophenacyl bromide) and restored by the addition of exogenous phospholipase A_2 activity (Tan et al, 1984). In simple phospholipid bilayer systems, such as those found in liposomes, lipid peroxidation cannot be prevented by GSH peroxidases unless phospholipase A_2 is incorporated into the system (Sevanian et al, 1983).

It is conceivable that phospholipase A_2, by releasing free fatty acid hydroperoxide, could alone prevent lipid peroxidation. However, the free fatty acid hydroperoxide is relatively non-polar and would have a tendency to remain in the membrane and retain its potential for membrane damage, were it not for the soluble GSH peroxidases (Tan et al, 1984).

Liver perfusion experiments have confirmed that both the selenium-independent and selenium-dependent peroxidases operate in vivo (Sies et al, 1972; Oshino and Chance, 1977; Burk et al, 1978). In normal rats glutathione disulfide is released into bile upon infusion of both H_2O_2 and t-butyl hydroperoxide. In selenium-deficient animals only t-butyl hydroperoxide is effective. The GSH S-T may therefore perform an important biological function in protecting the cell against the harmful effects of lipid peroxidation.

1.12. BIOTRANSFORMATION OF XENOBIOTICS IN THE FETUS

Biological development is a process that continues for much of the life-span of an animal. It is generally accepted that the developing embryo, fetus and neonate show the greatest susceptibility to developmental interference (Snell, 1985). In early embryonic life such interference results in fetal death or malformations (teratogenesis), whereas at the fetal and neonatal stages of development interference results in functional or metabolic disturbances ('metabolic teratogenesis') (Snell, 1985).

The fetal liver occupies a unique anatomical position, interposed between the umbilical vein and the inferior vena cava. All xenobiotics passing the placenta must thus traverse the fetal liver prior to entering the fetal systemic circulation (Green et al, 1979) and consequently the fetal liver may play an important role in the metabolism of transplacental xenobiotics. However, despite the importance, the enzyme systems responsible for the biotransformation of xenobiotics in the fetal liver have not been fully characterized. This section will describe the results of some of the previous studies conducted in this field.

1.12.1. Hepatic Oxidation Reactions

The fetal livers of many laboratory animal species, including the rat, mouse, guinea-pig, rabbit, hamster and ferret are deficient in hepatic, microsomal, xenobiotic-oxidizing activity associated with the cytochrome

P-450-linked microsomal electron transport chain (Rane et al, 1973; Kuenzig et al, 1974; Bend et al, 1975; Bell et al, 1975; Ecobichon et al, 1978; Nau and Neubert, 1978; Pelkonen, 1980; Brown and Manson, 1986). The appearance of the oxidative enzyme system in the liver takes place in the later stages of fetal development (Maydl and Metzler, 1984) and, depending on the species, adult levels are attained within a few days as in the case of the guinea-pig, or in a few weeks as in the case of the rat.

In contrast, human and primate fetal livers possess a relatively well developed mono-oxygenase complex capable of oxidizing a wide variety of xenobiotics (Pelkonen et al, 1973; Rane and Ackermann, 1972; Ackermann and Richter, 1977). The human enzyme system is already detectable in the fetus at the age of 6-7 weeks i.e. in the end of embryogenesis (Pelkonen and Karki, 1973).

1.12.2. Hepatic Hydrolytic Reactions

One of the few hydrolytic enzymes to be studied to any significant extent is epoxide hydratase, which is important in converting epoxides into dihydrodiols (Oesch, 1973). This enzyme can generally be regarded as a detoxifying enzyme as it converts reactive epoxides into usually less active dihydrodiols. However, it also plays an activating role in the diol-epoxide pathway of carcinogen activation (Jerina and Lehr, 1977). By detecting the in vitro formation of different dihydrodiols of benzo(a)pyrene, the presence of epoxide hydratase has indirectly been demonstrated in human fetal liver (Pelkonen and Karki, 1975; Pelkonen et al, 1975) and in hamster fetal livers. Using styrene oxide as a substrate, epoxide hydratase activity has been detected at very low levels in liver microsomes from rat fetuses (Oesch, 1975) as well as those from guinea-pig and rabbit fetuses (James et al, 1977).

1.12.3. Conjugation Reactions

Most conjugation pathways of xenobiotic metabolism are poorly developed in the fetal livers of any one species, but not as uniformly as xenobiotic-oxidation pathways (Pelkonen, 1980).

The development of glucuronidation has been shown to be dependent not only on the species but also on the strain and the substrate used to detect enzyme activity (Dutton and Burchell, 1977). Burchell (1974) found that in ASH/TO-strain mice, UDP-glucuronyltransferase activity towards o-aminophenol in the livers of fetuses which were 14 days old, was close to adult levels, whereas the activity of the same samples towards oestriol was less than 1% of the adult activity. This suggests that like the GSH S-T (Chapter 1, Section 1.8.1.), the development of this enzyme system is not homogeneous.

Bilirubin UDP-glucosyl-transferase activity (Wong, 1972) and glycine conjugation (Irjala, 1972) are low in the fetal livers of neonatal rodents. However, sulphate conjugation of 7-hydroxyacetyl-aminofluorene, diethylstilbestrol and morphine by fetal guinea-pig liver has been shown to be relatively high compared to maternal liver (Namkung et al, 1977).

1.12.4. Inducibility of Fetal Enzymes

Xenobiotic metabolizing enzymes in fetal liver and other tissues can be induced by perinatal exposure to foreign chemicals (Pelkonen, 1982). The majority of studies indicate that fetal microsomal enzyme activity can be induced only when the inducing agent is administered to the mother during the last few days of gestation.

Inducers like polycyclic aromatic hydrocarbons and polychlorinated biphenyls which induce cytochrome P-448 or P_1-450 (Thorgeirsson and Nebert, 1977) are the most potent inducers during the perinatal period, whereas phenobarbital and other inducers hardly induce xenobiotic metabolism (Guenthner and Mannering, 1977; Cresteil et al, 1979). Synergistic effects between inducing agents such as phenobarbital and 3-methylcholanthrene have also been observed (Guenthner and Mannering, 1977). In general, the absolute fetal enzyme activities reached after induction are much lower than they are in the neonatal or the adult liver, although the ratio between induced and basal values may be quite high because of low basal values. Xenobiotic metabolizing enzymes in the human fetal liver are inducible during late pregnancy by anticonvulsants (Pelkonen, 1980) but more studies are needed to reveal induction during early pregnancy.

1.12.5. Ontogenic Patterns

Studies have demonstrated that the prenatal and postnatal development of different enzyme activities may follow differing time courses (Klinger et al, 1979) and may be more complicated than previously thought. Henderson (1978) found no parallels in the rate of postnatal development between the oxidative N-demethylation of N-methyl and N,N-dimethyl-amphetamine in the rat liver. This suggests that multiple forms of N-demethylating enzymes exist that develop independently of each other. Wishart and Dutton (1978) have shown that there is a developmental heterogeneity of UDP-glucuronyltransferase activity towards two groups of substrates which they have classified as the 'late fetal group' and the 'neonatal group' on the basis of the time of developmental upsurge. Snell (1985) has extrapolated this further by suggesting that the development of liver enzymes during terminal differentiation may be classified into three discrete stages, namely, fetal, neonatal and weanling clusters.

In the neonatal rabbit aryl hydrocarbon hydroxylase activity has been shown to be induced by 3-methylcholanthrene or 2,3,7,8-tetrachlorodibenzo-p-dioxin and this induction is associated with the appearance of a specific form of cytochrome P-450 as evidenced by an additional band seen on SDS-polyacrylamide gel electrophoresis in the region of cytochrome P-450 proteins (Atlas et al, 1977). Further studies have shown that a similar kind of perinatal development can be detected in the rat and mouse (Guenthner and Nebert, 1978).

1.12.6. Generation of Reactive Metabolites by Fetal Tissues

In vitro studies have shown that fetal tissues are able to generate reactive metabolites of various xenobiotics. Sehgal and Hutton

(1977) have shown that rat fetal tissues catalyze the formation of carcinogenic metabolites of 2-aminoanthracene which are mutagenic to Salmonella typhimurium strains. The administration of 3-methyl-cholanthrene to pregnant rats increased the mutagenic activity. Similarly, rat and mice fetal tissues have been shown to be capable of converting 7,12-dimethyl-benz(a)anthracene, benzo(a)pyrene and 2-acetyl-amino-fluorene into mutagenic metabolites (Juchau et al, 1979). The above evidence is circumstantial and does not provide direct proof that fetal tissues catalyze the formation of reactive metabolites in vivo. However, experimental studies on transplacental carcinogenesis have suggested that agents requiring metabolic activation are effective just prior to birth with the appearance of xenobiotic metabolizing enzymes in fetal tissues (Pelkonen, 1982). This suggests that xenobiotic metabolizing enzymes may serve an important detoxification role in the later stages of fetal development.

PART B

EXPERIMENTAL

CHAPTER 2

PURIFICATION OF THE MAJOR BASIC RAT LIVER GLUTATHIONE S-TRANSFERASES

2.1. INTRODUCTION

The aim of this thesis was to look at the expression of the GSH S-T in fetal rat livers. However, before this could be attempted, for reference and comparison purposes, it was necessary to purify the major basic rat liver GSH S-T isoenzymes.

Normal adult rat livers contain six major cationic GSH S-T isozymes (see Chapter 1, Section 1.4.1.). These dimeric proteins are composed of subunits termed Y_a, Y_b, $Y_{b'}$ and Y_c. Three of the six isozymes (two homodimers and one heterodimer), are composed of subunits Y_a and Y_c while the remaining three isozymes consist of similar combinations of subunits Y_b and $Y_{b'}$. These major isoenzymes were purified using a combination of S-hexylglutathione affinity chromatography, carboxymethyl-cellulose cation exchange chromatography and chromatofocusing.

2.2. MATERIALS AND METHODS

2.2.1. Chemicals

All chemicals used were of analytical grade. The ion exchanger CM52 was purchased from Whatman. Chromatofocussing resin PBE118, and ampholines (pH 8-10.5) were from Pharmacia. Ampholines used for gel isoelectric focusing were from LKB. Freund's adjuvant was from Difco Laboratories (Detroit, Michigan). 1-Chloro-2,4-dinitrobenzene (CDNB) was from the Sigma Chemical Company.

S-Hexylglutathione was synthesized by method A of Vince et al (1971). 2 mmoles of GSH were dissolved in 2 ml H_2O. 4 mmoles of 2 M NaOH was added with stirring at room temperature. Ethanol was slowly added during constant stirring to the "cloud point" (12-15 ml), after which equimolar (2 mmoles) 1-iodohexane was gradually added, with vigorous stirring, over a period of 30 min. The mixture was then stirred at room temperature overnight. The pH was then reduced to 3.5 by the dropwise addition of 47% hydriodic acid (approximately 0.75 ml) and the solution was then chilled to 4°C. The precipitated S-hexylglutathione was separated by filtration and washed with cold water. Recrystallisation was carried out and the sample was dried and stored in a dessicator at 4°C.

2.2.2. Preparation of S-Hexylglutathione Affinity Matrix

Epoxy-activated Sepharose 6B (Pharmacia) was coupled with ligand essentially as prescribed by the manufacturer. For preparation of the

S-hexylglutathione affinity matrix, 6g of epoxy-activated Sepharose 6B was swollen in deionized water and washed on a sintered glass filter with 800 ml H_2O for 60 min. 94 mg of S-hexylglutathione was dissolved in 12 ml deionized water and adjusted with 2 M NaOH to a pH of approximately 12. The ligand solution was then added to the gel suspension and coupling was allowed to proceed for 16 hours at 30°C with gentle shaking. Excess ligand was washed from the gel with deionized water on a sintered glass filter. The gel was then treated with 50 ml of 2 M ethanolamine (pH 9.0) for 4 hours at 30°C with gentle shaking. After additional washing with deionized water, the gel was treated with each of the following in turn, 200 ml 0.1 M sodium acetate (pH 4.0)/0.5 M NaCl, and 200 ml 0.1 M Tris-HCl buffer (pH 8.0)/0.5 M NaCl. The treatment with these buffers was repeated three times followed by a final wash with 400 ml H_2O. The gel was then stored at 4°C in the presence of 0.02% (w/v) sodium azide to prevent microbial growth.

2.2.3. Assay of GSH S-Transferase Activity

The standard assay used during purification was based on the reaction between 1 mM GSH and 1 mM CDNB (ϵ = 9600 $M^{-1}.cm^{-1}$) in a 3 ml system at 25°C (Habig et al, 1974b). The activity was measured on a spectrophotometer at 340 nm, with reaction rates being corrected for the small amount of non-enzyme catalysed conjugation. Enzyme activity (µmol/min per mg) was calculated from the formula :

$$\frac{\text{OD/min}}{(\epsilon)} \quad \times \quad \frac{\text{final assay vol.}}{\text{sample vol.}} \quad \times \quad \frac{10^3}{\text{protein concn. (mg/ml)}}$$

The reaction mixture, in a cuvette, consisted of 2.75 ml 0.1 M potassium phosphate buffer (pH 6.5); 0.1 ml CDNB (30 mM in 95% ethanol) and 0.15 ml GSH (20 mM in H_2O). Enzyme source (0.01-0.05 ml) was added to the above mixture and the rate of conjugate formation was measured on the spectrophotometer. A blank contained all of the above with enzyme source replaced by buffer identical to that of the protein solution.

2.2.4. Protein Determinations

Protein concentrations were measured by the procedure of Bradford (1976), standardized to bovine serum albumin (Miles Laboratories).

2.2.5. Gel Electrophoresis

Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulphate (SDS) was performed on vertical slab gels (Laemmli, 1970). The system comprised a 3% (w/v) stacking gel (approximately 3 x 14 x 0.2 cm) in 0.14 M Tris-HCl buffer (pH 6.8), and a 7.5 to 17.5% (w/v) gradient gel (20 x 14 x 0.2 cm) in 0.56 M Tris-HCl buffer (pH 8.8). Samples were prepared as described by Maizel (1971). Gels were fixed and stained for 2.5 hours in a methanol/water (1:2, v/v) solution containing 10% trichloroacetic acid and 1% Coomassie Brilliant Blue R. Destaining was carried out with a 5% methanol/7% acetic acid (v/v) solution. Protein standards used were phosphorylase b (M_r 94 000), albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), trypsin inhibitor (M_r 20 100), and alpha-lactalbumin (M_r 14 400). The relative mobility (R_f) of each protein was calculated from the formula :

$$R_f = \frac{\text{distance protein has migrated from origin}}{\text{distance from origin to reference point}}$$

Plots of relative mobility (R_f) versus log M_r showed a linear relationship, and were used for the determination of unknown M_r values.

2.2.6. **Gel Isoelectric Focusing**

Gel isoelectric focusing was performed at 4°C essentially as described by Wrigley (1972). The gels (8 x 0.3 cm) comprised 10% polyacrylamide with 2% (w/v) ampholines (pH 3.5-10/pH 9-11, 2:1). Gels were pre-focused at 1 mA/gel for 45 min. Protein samples (2.5-20 µg in H_2O) containing 0.1% ampholines were then loaded onto gels and focused at 200 V for 15 min, and subsequently at 400 V for 16 hours. Gels were washed with hourly changes in 5% trichloroacetic acid over a period of 5 hours to remove carrier ampholytes. Staining and destaining were carried out as described under Section 2.2.5. The pH gradient was determined by cutting two of the gels, identical to those to be stained, into 2 mm sections. These sections were then eluted in 0.5 ml H_2O by constant agitation at room temperature for 2 hours. The pH of the eluate was measured and a graph of pH gradient versus gel length, drawn.

The distances migrated by stained bands were then measured and multiplied by a correction factor determined as follows :

$$\text{Correction factor} = \frac{\text{prestaining length}}{\text{post-staining length}}$$

The pI of stained protein bands could then be determined from the graph.

2.2.7. Preparation of Antisera

Male New Zealand White rabbits were immunized with an initial innoculation consisting of 2.0 ml of equal volumes of protein antigen 0.05 mg/ml in 0.01 M sodium phosphate buffer (pH 7.4)/0.15 M NaCl and complete Freund's adjuvant. The innoculum was introduced into multiple subcutaneous sites on the back of the animal as well as intranodally. Subsequent subcutaneous booster innoculations, substituting incomplete Freund's for the complete, were administered fortnightly. After two innoculations animals were bled and tested for antisera production.

2.2.8. Immunodiffusion

Immunodiffusion studies were performed as described by Ouchterlony (1958). 1.2 g Agar-Noble, 0.1 g sodium azide and 100 ml of 0.05 M sodium phosphate buffer (pH 7.4)/0.15 M NaCl were heated in a conical flask until the solution clarified and kept molten in a water bath set at 60°C. 10 ml of the molten agar solution was then evenly poured onto horizontally positioned warmed glass plates (9.5 x 8.2 cm). Following a cooling period of approximately 60 min, wells (4 mm in diameter) were cut into the agar using an LKB 2117 Multiphor template and bore. 10 µl of antiserum was placed into the central well and 10 µl of antigen into the peripheral wells. The plates were kept in an air-tight moisture box at room temperature for 48 hours, after which they were washed for 3 days with several changes of a solution comprising 0.9% NaCl/0.1% sodium azide, to remove non-precipitated protein. Plates were stained for 3 hours in a 5% acetic acid solution containing 1% Amido Black, and destained in a 5% acetic acid solution.

2.2.9. Immunoelectrotransfer Blot Technique ("Western Blot")

This was performed at 4°C using a modification of the method described by Tsang et al (1983). The apparatus comprised a TE 52 Transfor Electrophoresis Cell buffer chamber (Hoeffer Scientific Instruments, San Francisco) and the associated TE 50 Hoeffer Power lid. Six pieces of Whatmann chromatography paper (14 x 20 cm), an equi-sized piece of nitrocellulose paper (Schleicher and Schuell, Dassel, W. Germany) and the Scotch-Brite pad of the blotting cassette were pre-soaked in transfer buffer (pH 8.3) comprising 0.02 M Tris, 0.153 M glycine and 20% methanol (v/v). The Scotch-Brite pad was placed on top of an open half of the blotting cassette and three pieces of the pre-soaked chromatography paper were in turn placed on top of the pad. The nitrocellulose paper was placed on top of the chromatography paper and the polyacrylamide gel to be blotted was in turn placed on top of the nitrocellulose paper, care being taken to avoid the trapping of air bubbles between the two surfaces. The position of the outer edges of the gel were marked on the nitrocellulose paper for orientation purposes. The final three pieces of chromatography paper were positioned on top of the gel and the surface rolled with a section of glass tubing to ensure the removal of all air bubbles. The remaining half of the blotting cassette was placed on top of these layers and clipped into place to complete the "sandwich". The buffer chamber was placed inside a polystyrene container and the space around the outer surfaces of the chamber were packed with ice. The chamber was filled with approximately 5 litres of transfer buffer, pre-cooled to 4°C, and the "sandwich" was slowly lowered into its place in the chamber, with the nitrocellulose side toward the positive electrode. Electrophoretic transfer was carried out at 95 V (approximately 350 mA) for 1 hour. Blotting was performed at 4°C to preserve the solution and extend the life of the medium.

Following blotting, the nitrocellulose paper was placed into 100 ml of 0.01 M Tris-HCl buffer (pH 7.8)/0.15 M NaCl containing 3% bovine serum albumin (buffer A) for 1 hour. This was performed at room temperature, with constant shaking, and is effective in blocking nonspecific binding sites on the paper. Thereafter the nitrocellulose paper was placed into 50 ml buffer A containing 100 µl of specific antiserum and shaken for a further hour. The paper was then given six, 10 min, washes with 0.15 M NaCl and placed into 50 ml of buffer A containing 100 µl peroxidase-conjugated goat anti-rabbit immunoglobulins (Behringwerke AG, Marburg, W. Germany) and allowed to incubate for 1 hour with constant shaking. The six washes with

0.15 M NaCl were again repeated, followed by a final rinse with 0.01 M Tris-HCl buffer (pH 7.4). The paper was then either :

(i) added to a solution comprising 24 mg of 4-chloro-1-naphthol (Sigma Chemical Company), 40 ml Tris-HCl (pH 8.0)/0.15 M NaCl, 0.01 M glycine and 0.04% sodium azide, or

(ii) added to a solution comprising 0.1 mg 3,3'-diamino-benzidine tetrahydrochloride (BDH Chemicals Ltd.) in 40 ml 0.01 M Tris-HCl buffer (pH 7.4).

15 µl hydrogen peroxide was finally added which allowed visualization of protein bands. Following the required degree of development, the paper was washed with deionised water and allowed to dry in the dark.

2.2.10. Preparation of Cytosol Fraction

The liver (7-10 g) from a male Long Evans rat was excised and immediately perfused via the hilar vessels with ice-cold 0.01 M Tris-HCl buffer (pH 7.8). Initial fragmentation was performed with scissors, followed by homogenisation in sufficient of the Tris-HCl buffer to produce a 25% homogenate. All steps were carried out at 4°C. Initial centrifugation was at 27 000 x g for 30 min in a Sorvall RC-5 superspeed centrifuge. The supernatant fraction was carefully removed without disturbing the floating lipid layer and re-centrifuged at 100 000 x g for 120 min in a Beckman model L5-65 ultracentrifuge. The supernatant was once again removed and stored on ice.

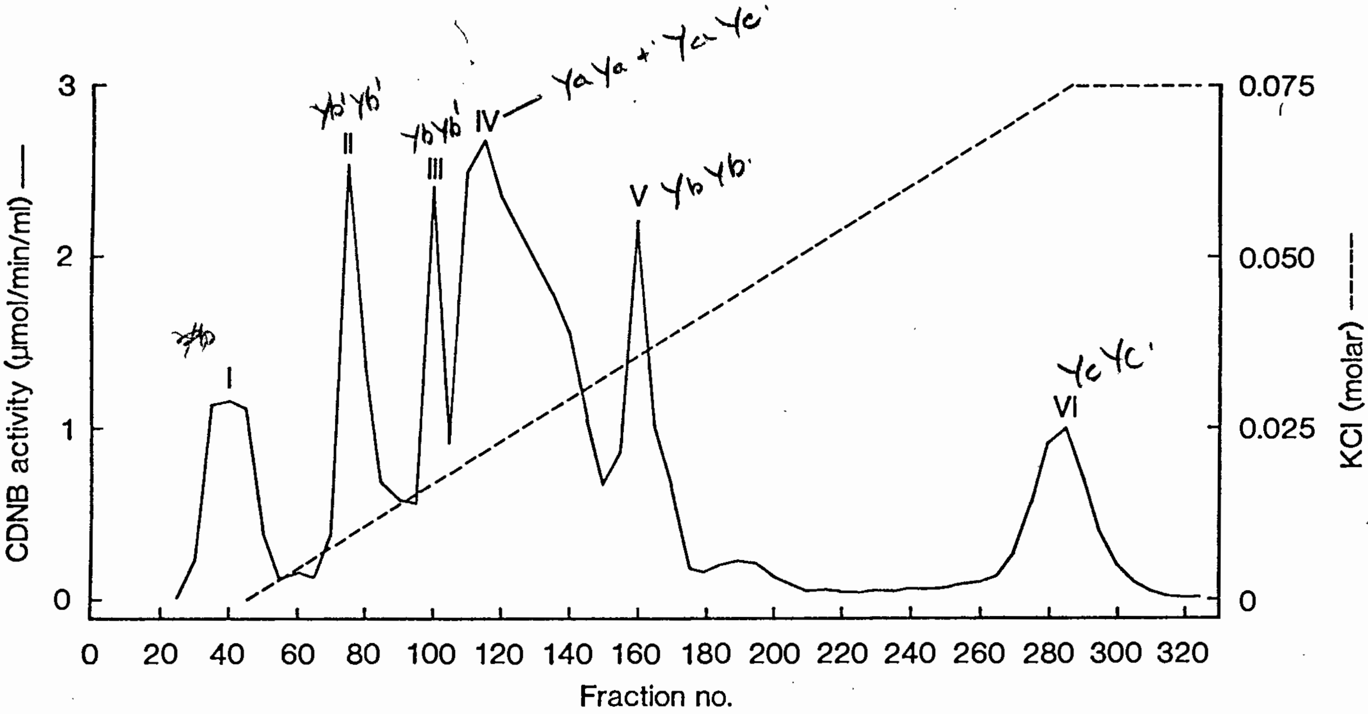
2.2.11. GSH S-Transferase Purification

A 50-ml aliquot of the cytosol fraction was chromatographed on a Sephadex G-25 (Pharmacia) column (3.2 x 60 cm) previously equilibrated with 0.01 M Tris-HCl buffer (pH 7.8) (buffer A). Fractions exhibiting GSH S-T activity with CDNB as substrate were pooled and applied to an affinity column (1.6 x 15 cm) of S-hexylglutathione linked to epoxy-activated Sepharose 6B, equilibrated with buffer A. The column was washed with 200 ml 0.01 M Tris-HCl (pH 7.8)/)0.2 M NaCl, after which the GSH S-T were eluted with 5 mM S-hexylglutathione in the same buffer. Fractions showing absorption at 280 nm were pooled, concentrated with stirring over an Amicon PM 10 membrane, and applied to a Sephadex G-25 column (1.6 x 60 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 6.7)(buffer B). Fractions exhibiting GSH S-T activity with CDNB were pooled and applied to a carboxymethyl-cellulose (CM52) cation-exchange column (1.6 x 15 cm) equilibrated with buffer B. The column was washed with approximately 120 ml buffer B and then developed with a linear salt gradient of 0-75 mM KCl in 0.01 M buffer B in a total volume of 720 ml of buffer. Fractions of 3 ml were collected and assayed for transferase activity. The six major peaks were then in turn each pooled, concentrated, and applied to a Sephadex G-25 column (1.6 x 60 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.0). Fractions exhibiting GSH S-T activity were pooled, concentrated, and applied to a PBE118 chromatofocusing column (1.0 x 30 cm) equilibrated with 25 mM triethylamine-HCl buffer (pH 11.0). Elution was carried out with 500 ml of Pharmalyte (pH 8-10.5), diluted 1:80, and adjusted with HCl to pH 8.0. Fractions of 2 ml were collected and assayed for transferase activity. Those fractions containing purified enzyme were pooled and subjected to Sephadex G-75 chromatography to remove the ampholines. The purified enzyme in 0.01 M sodium phosphate buffer (pH 7.4)/0.15 M NaCl was frozen at -70°C until used.

2.3. RESULTS AND DISCUSSION

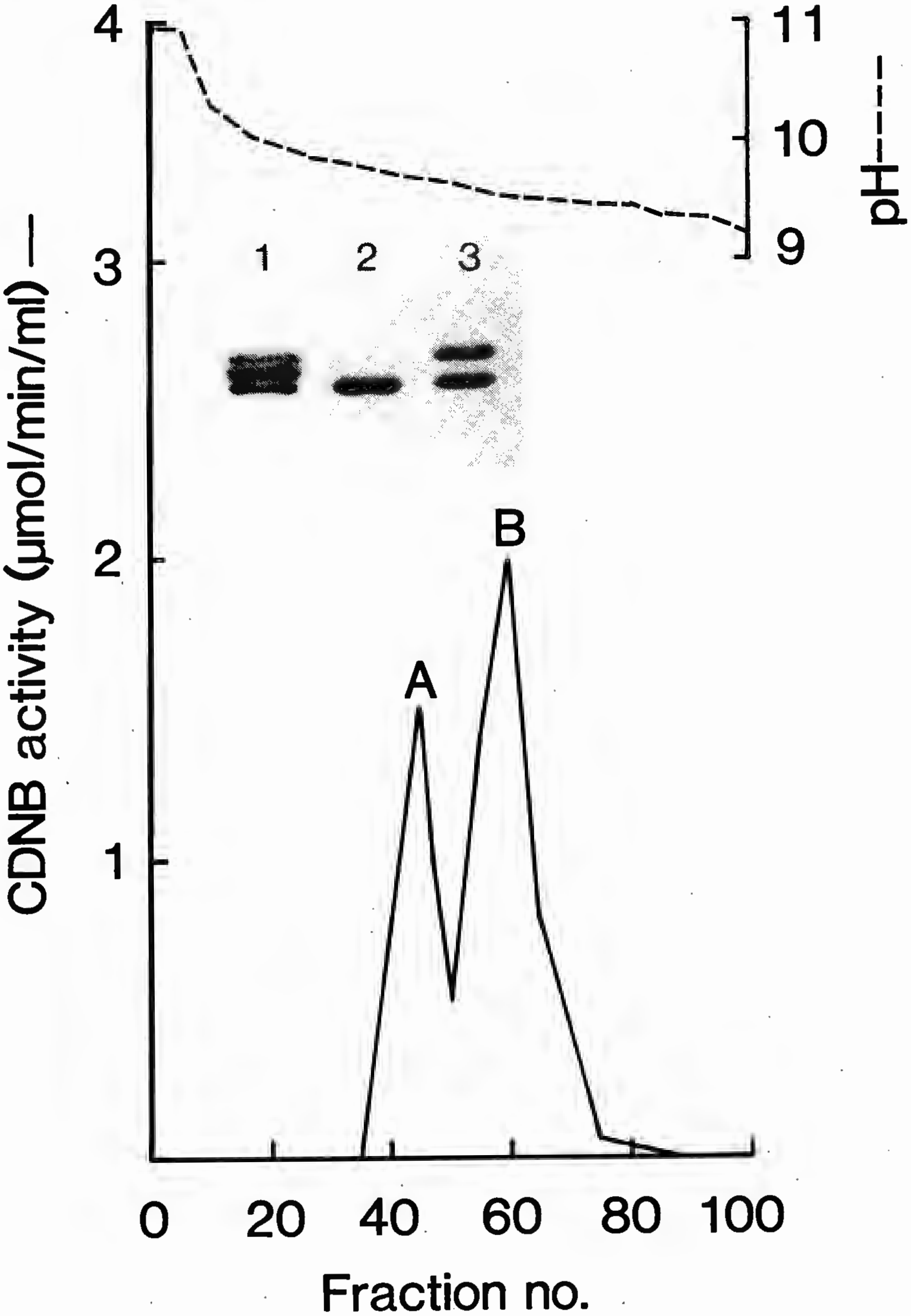
Following S-hexylglutathione affinity chromatography, six major peaks of GSH S-T activity (peaks I-VI) were resolvable by carboxymethyl-cellulose chromatography (Fig. 2.1). This profile is similar to that obtained using the 'standard' purification scheme previously employed for these isoenzymes (Jakoby et al, 1976; Habig and Jakoby, 1981). This 'standard' method excludes S-hexylglutathione affinity chromatography but includes DEAE-cellulose and hydroxylapatite chromatography. Peak I was eluted before the application of a salt gradient and comprises acidic isoenzymes which do not bind to the carboxymethyl-cellulose column. Peaks II-VI corresponded to GSH S-T D ($Y_{b'}Y_{b'}$), C ($Y_bY_{b'}$), B (Y_aY_a and Y_aY_c), A (Y_bY_b), and AA (Y_cY_c) respectively (Habig and Jakoby, 1981).

Peaks II-VI were each in turn further purified by chromatofocusing on a PBE118 column to seperate possible isoenzymes according to their isoelectric points. Peaks II, III, V and VI could not be further purified. However, peak IV (GSH S-T B) could be further resolved into two peaks (Fig. 2.2). These peaks eluted in the pH range 9.3-9.8. On SDS-polyacrylamide gel electrophoresis peak A comprised one band with a M_r of 25 000, which identified it as GSH S-T Y_aY_a while peak B comprised two subunits with M_r values of 25 000 and 28 000 which identified it as GSH S-T Y_aY_c. Fig 2.3 shows the subunit composition of peaks II, III, V and VI, following chromatofocusing and G-75 chromatography.



2.3.

Fig. 2.1. **Resolution of adult rat liver GSH S-T isoenzymes by carboxymethyl-cellulose column chromatography. The activity was measured with CDNB and GSH as substrates.**



2.5
Fig. 2.2. Chromatofocusing of peak IV obtained by carboxymethyl-cellulose column chromatography. The insets show the subunit composition of peak A (lane 2) and peak B (lane 3) on SDS-polyacrylamide gel electrophoresis. Lane 1 of the inset shows the subunit composition of the GSH S-T derived from adult rat livers by affinity chromatography.

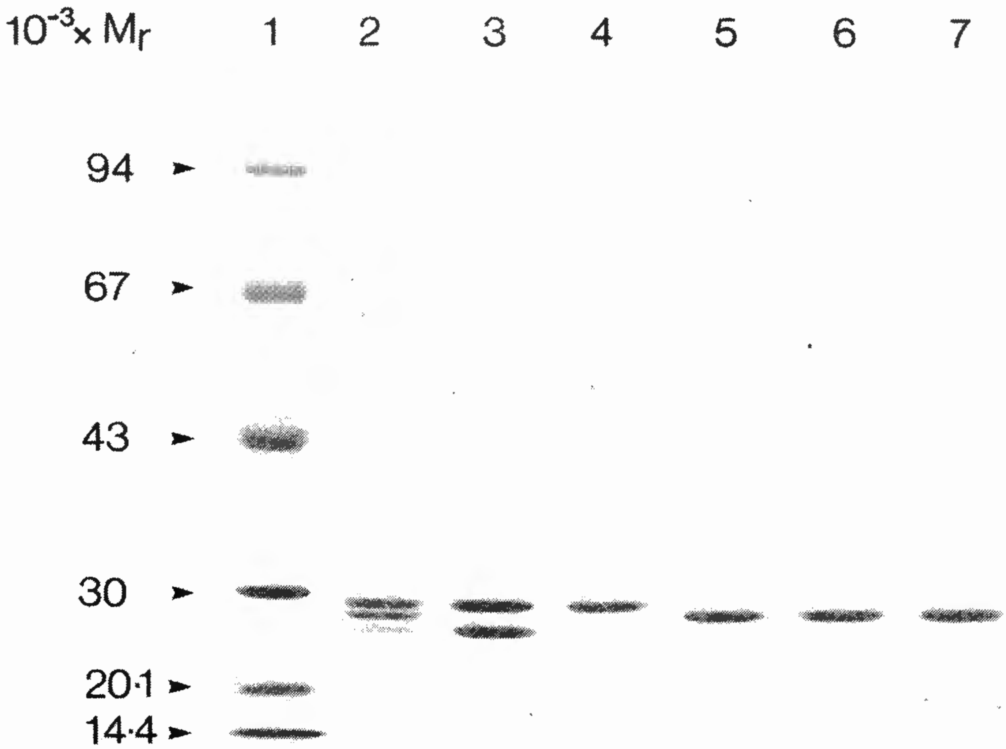


Fig. 2.~~3~~b. **SDS-polyacrylamide gel electrophoresis showing the subunit composition of carboxymethyl-cellulose peaks II, III, V and VI following chromatofocusing and G-75 chromatography. Lane 1 shows standard molecular weight markers; lane 2 shows the subunit composition of the GSH S-T derived from adult rat livers by affinity chromatography; lane 3 shows GSH S-T Y_aY_c as reference; lane 4 shows Y_cY_c (peak VI); lane 5 shows Y_bY_b (peak V); lane 6 shows $Y_bY_{b'}$ (peak III); and lane 7 shows $Y_{b'}Y_{b'}$ (peak II).**

Gel isoelectric focusing confirmed the identities of GSH S-T Y_bY_b (peak V), Y_bY_b' (peak III) and Y_b'Y_b' (peak II). Table 2.1 lists the pH at which these isoenzymes eluted from a chromatofocusing column, as well as their isoelectric points and apparent M_r values. The elution pH from the chromatofocusing column was found to differ from the isoelectric point of the protein. This has previously been reported by others (Alin et al, 1985b).

Immunodiffusion was used to confirm the presence of antibodies in the serum of rabbits injected with the purified proteins. The specificty of the antiserum to injected antigen was confirmed by "Western Blot" in which the antiserum was checked for cross-reactivity with the other purified isoenzymes. Table 2.2 lists the immunochemical properties of the six major GSH S-T isoenzymes.

The specific activities of the six isoenzymes to various electrophilic substrates, and which do not differ significantly from those obtained by others under similar conditions, are given in Chapter 4 (Table 4.3).

In summary, the purification yielded six basic rat liver GSH S-T isoenzymes which on the basis of physical, chemical and immunochemical properties, are apparently identical with the six major rat liver GSH S-T isoenzymes previously described in the literature (Jakoby et al, 1976; Habig and Jakoby, 1981; Mannervik, 1985a; Alin et al, 1985b).

Table 2.1. **PHYSICAL PROPERTIES OF THE SIX MAJOR GSH S-TRANSFERASES ISOLATED FROM ADULT RAT LIVERS**

Properties	Isoenzyme					
	Y_aY_a	Y_aY_c	Y_cY_c	Y_bY_b	$Y_bY_{b'}$	$Y_{b'}Y_{b'}$
CM-cellulose peak	IV	IV	VI	V	III	II
Subunit M_r	25K	25K + 28K	28K	26.5K	26.5K	26.5K
PBE118 elution pH	9.7	9.5	9.7	8.9	8.7	8.2
Isoelectric point*				8.4	8.1	7.0

*The isoelectric points of GSH S-T Y_aY_a, Y_aY_c and Y_cY_c were not determined as their identities could be ascertained from their subunit composition on SDS-polyacrylamide gel electrophoresis.

Table 2.2. **IMMUNOCHEMICAL PROPERTIES OF THE SIX MAJOR GSH S-TRANSFERASES ISOLATED FROM ADULT RAT LIVERS**

Reaction with Antiserum to Isoenzyme	Isoenzyme					
	Y_aY_a	Y_aY_c	Y_cY_c	Y_bY_b	$Y_bY_{b'}$	$Y_{b'}Y_{b'}$
Y_aY_a	+	+	-	-	-	-
Y_aY_c	+	+	+	-	-	-
Y_cY_c	-	+	+	-	-	-
Y_bY_b	-	-	-	+	+	-
$Y_bY_{b'}$	-	-	-	+	+	+
$Y_{b'}Y_{b'}$	-	-	-	-	+	+

CHAPTER 3

THE IDENTIFICATION OF A GLUTATHIONE S-TRANSFERASE ISOZYME IN FETAL RAT LIVERS WHICH IS ABSENT FROM ADULT LIVERS

3.1. INTRODUCTION

Little is currently known about the GSH S-T composition of fetal rat livers (Chapter 1, Section 1.8.1.). Since GSH S-T subunits differ in their affinity for various substrates (Chapter 1, Section 1.3.2.) the presence or absence of a particular subunit or inter-individual variation in the expression of a subunit, could have important fetotoxic implications.

This chapter will describe an investigation into the subunit expression of the GSH S-T in fetal rat livers, and report the presence of a previously undescribed transferase subunit.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals

All chemicals used were of analytical grade (see Chapter 2, Section 2.2.1.).

3.2.2. Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed as previously described (Chapter 2, Section 2.2.5.). Gel isoelectric focusing was carried out using 10% polyacrylamide gels (Chapter 2, Section 2.2.6.)

3.2.3. Western Blotting

Western blotting (Chapter 2, Section 2.2.9.) was performed using antibodies raised in rabbits against the major basic rat liver GSH S-T purified as described in Chapter 2 (Section 2.2.11.)

3.2.4. Animals

Rats of the Long Evans strain were used throughout this study. Timed pregnancies were achieved by placing 2-3 month old proestrus female rats overnight (4 p.m. to 9.30 a.m.) with males of the same strain. The presence of spermatozoa in vaginal smears was taken as an indication of successful copulation, and the date was fixed as day zero of gestation.

3.2.5. Preparation of Cytosol Fraction

Livers obtained from seventeen or eighteen day fetuses and their respective dams were immediately rinsed with ice-cold 0.01M Tris-HC1 buffer (pH 7.80)(buffer A) and a 25% homogenate was prepared using the same buffer. All steps were carried out at 4°C. Initial centrifugation was at 27 000 x g for 30 min. The supernatant fraction was then carefully removed and re-centrifuged at 100 000 x g for 120 min, following which the supernatant fraction was once again removed without disturbing the floating lipid layer. The cytosolic fraction from rat placentas was similarly obtained. All samples not used immediately were stored at -70°C.

3.2.6. Subunit Purification

The GSH S-T isozyme subunits from fetal rat livers, adult rat livers and rat placentas were purified by the same procedure. A 50-ml aliquot of the cytosol fraction was chromatographed on a Sephadex G-25 (Pharmacia) column (3.2 x 60 cm) previously equilibrated with buffer A. Fractions exhibiting GSH S-T activity with 1-chloro-2,4-dinitrobenzene as substrate (Chapter 2, Section 2.2.3.) were pooled and applied to an affinity column (1.6 x 15 cm) of S-hexylglutathione linked to epoxy-activated Sepharose 6B (Chapter 2, Section 2.2.2.), equilibrated with buffer A. The column was washed with 200 ml of 0.01 M Tris-HCl (pH 7.8)/0.2 M NaCl, after which the GSH S-T were eluted with 5 mM S-hexylglutathione in the same buffer. Fractions showing absorption at 280 nm were pooled and dialysed against buffer A to remove the S-hexylglutathione.

3.3. RESULTS AND DISCUSSION

Fig. 3.1 shows the subunit composition of the GSH S-T derived from fetal and adult rat livers by affinity chromatography. Placental GSH S-T was used as a reference (lane 2). In adult livers (lane 3) three protein bands, respectively identified on the basis of their apparent molecular weights as subunit Y_a (M_r = 25 000), subunits Y_b and $Y_{b'}$ (M_r = 26 500) and subunit Y_c (M_r = 28 000), were visible. In fetal livers (lane 4) there was an additional subunit (indicated by an arrow) which migrated between subunit Y_a and subunits $Y_b/Y_{b'}$, giving an apparent M_r value of 25 500.

Densitometric scanning (Zeiss Densitometer) of the Coomassie blue-stained bands revealed that the fetal transferase accounted for approximately 26% of fetal liver GSH S-T. The $Y_b/Y_{b'}$ subunits were the most prevalent subunits in fetal liver; the Y_a subunit being the least prevalent.

In order to determine whether the novel subunit found in fetal livers arose from the degradation of other GSH S-T, fetal GSH S-T subunits were allowed to stand at room temperature for 16 hours prior to SDS-polyacrylamide gel electrophoresis and densitometric scanning. Under these conditions, known to favour degradation of the GSH S-T, the subunit ratio didn't change.

The fetal transferase clearly differed from that of placenta (Fig. 3.1). Coelectrophoresis of the placental and fetal GSH S-T confirmed this finding (Fig. 3.2).

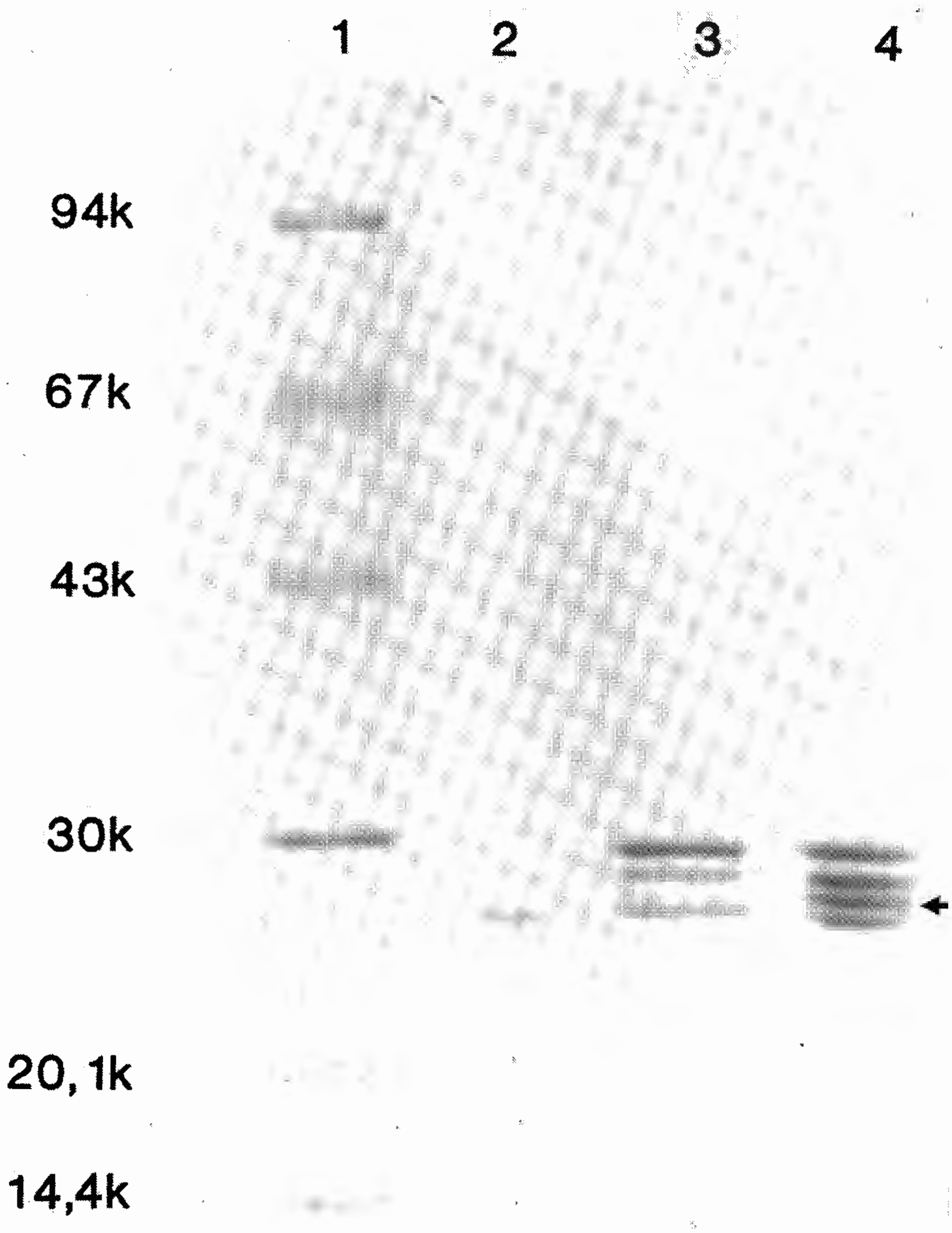


Fig. 3.1. SDS-polyacrylamide gel electrophoresis of molecular weight markers (lane 1), placental GSH S-T (lane 2), adult rat liver GSH S-T (lane 3) and fetal rat liver GSH S-T (lane 4). The additional subunit present in fetal liver is indicated by an arrow.

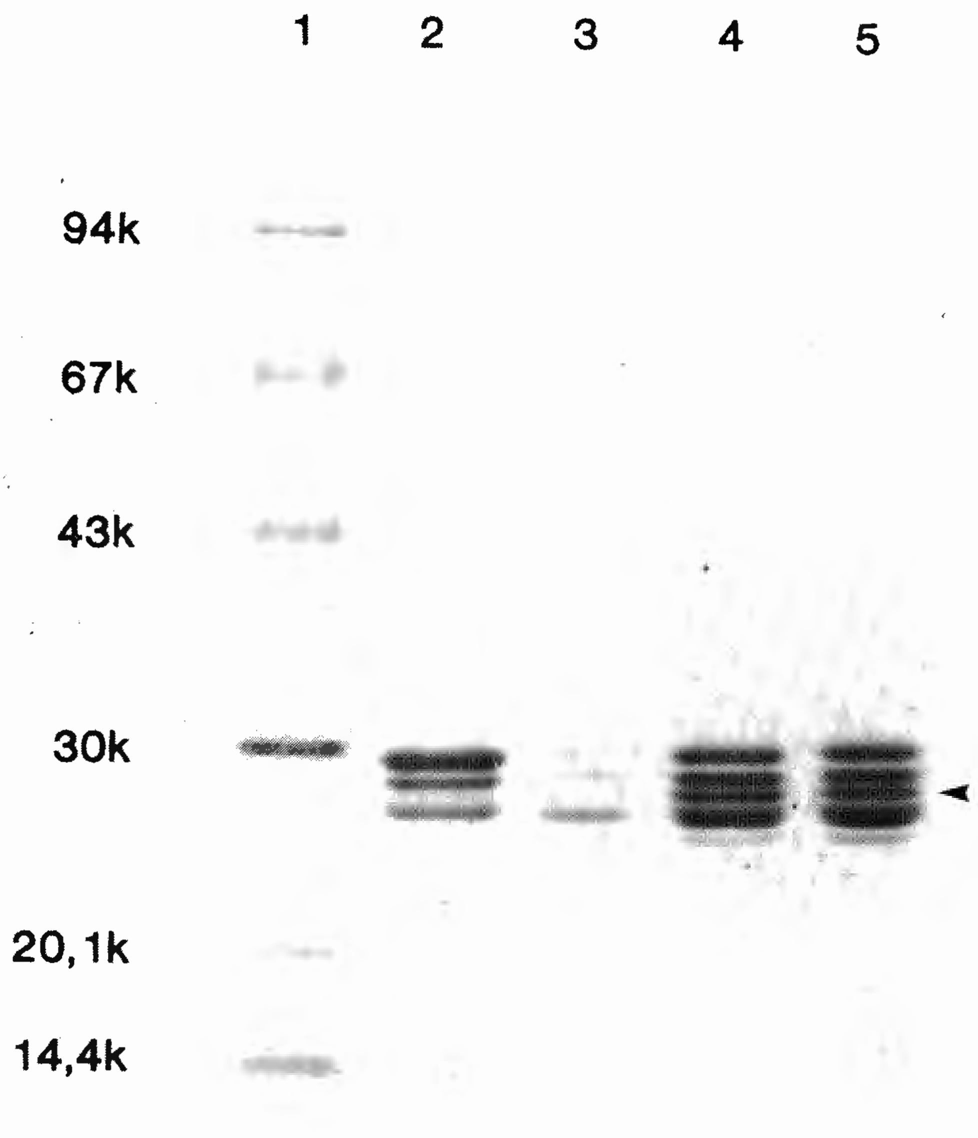


Fig. 3.2. SDS-polyacrylamide gel electrophoresis of molecular weight markers (lane 1), adult rat liver GSH S-T (lane 2), placental GSH S-T (lane 3), fetal rat liver GSH S-T (lane 4) and coelectrophoresis of rat placenta and fetal liver GSH S-T (lane 5). The additional subunit present in the fetal liver is indicated by an arrow. The low molecular weight band present in lane 5 is the placental subunit Y_p.

The possibility that proteases present in fetal liver cytosol might be responsible for the breakdown of subunits Y_c, Y_b or $Y_{b'}$ to produce the fetal subunit was investigated by mixing equal quantities of adult liver cytosol (25% homogenate, w/v) with fetal liver cytosol (25% homogenate, w/v) from which the GSH S-T had been removed using S-hexylglutathione affinity chromatography. The mixture was incubated overnight at 4°C and then applied to a S-hexylglutathione affinity column. After elution the GSH S-T were examined by SDS-polyacrylamide gel electrophoresis. This failed to produce the subunit present in fetal livers.

"Western blotting" using antibodies raised against the adult rat liver GSH S-T indicated that the unique fetal subunit reacted with antibodies raised against GSH S-T Y_aY_c and Y_cY_c (Fig. 3.3) but not GSH S-T Y_aY_a, Y_bY_b, $Y_bY_{b'}$ or $Y_{b'}Y_{b'}$. Immunodiffusion of adult and fetal GSH S-T against antisera to GSH S-T Y_aY_c and Y_cY_c suggested that this identity was incomplete (Fig. 3.4).

The distinctness of the fetal subunits was confirmed by isoelectric focusing; two bands with pI values of 8.0 and 8.5 were detected in fetal preparations and were clearly absent from adult rat livers.

In summary, the studies suggest that the additional band seen on SDS-polyacrylamide gel electrophoresis represents a fetal isoenzyme bearing antigens shared with the known adult subunits. This confirms the postulate that fetal forms of the GSH S-T may exist (see Chapter 1, Section 1.8.) and is of considerable interest in view of the reported restriction in the capacity of the fetus to metabolize certain xenobiotics (see Chapter 1, Section 1.12.).

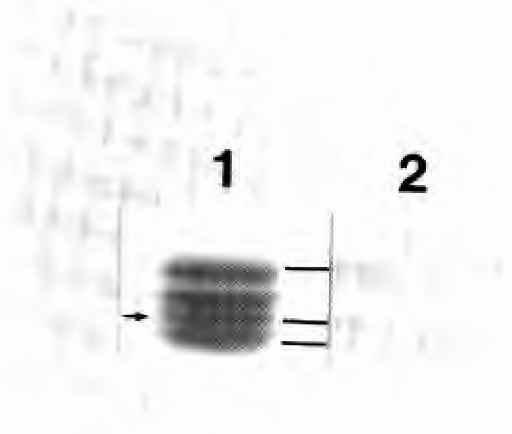


Fig. 3.3. "Western Blot" of rat fetal liver GSH S-T subunits using antiserum against GSH S-T Y_aY_c. The GSH S-T subunits of the fetal liver are shown in lane 1 and the respective "Western Blot" in lane 2. The additional subunit in the fetal liver is indicated by an arrow.

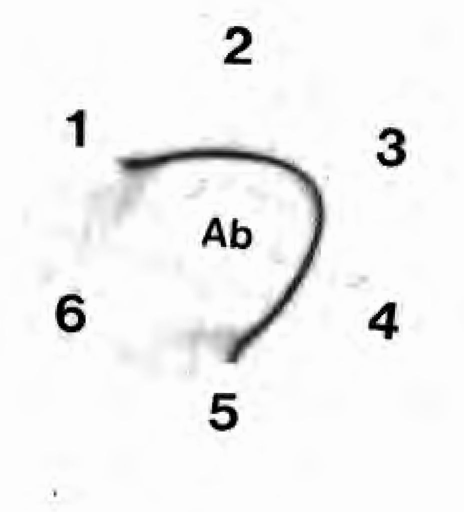


Fig. 3.4. Double immunodiffusion of antiserum to GSH S-T Y_aY_c against the GSH S-T of fetal and adult rat livers following their elution from an S-hexylglutathione affinity column. The centre well contains antibody to GSH S-T Y_aY_c. Wells 1, 3 and 5 contain rat fetal liver GSH S-T and wells 2 and 4 adult liver GSH S-T. Well 6 contains no antigen.

CHAPTER 4

THE ISOLATION OF A FETAL RAT LIVER GLUTATHIONE S-TRANSFERASE ISOENZYME WITH HIGH GLUTATHIONE PEROXIDASE ACTIVITY

4.1. INTRODUCTION

Chapter 3 described the existence of a major additional GSH S-T subunit and hence a further isoenzyme(s) in fetal rat livers which is absent from adult livers. This chapter will describe the purification and characterization of the major isoenzyme containing this subunit.

The limiting factor in the purification of this isoenzyme was the availability of fetal tissue. For this reason initial attempts at purification focused on the use of electrophoretic techniques which require relatively small amounts of protein. However, extensive attempts to purify the fetal isoenzyme using sucrose gradient isoelectric focusing proved unsuccessful because its isoelectric point was very similar to that of GSH S-T Y_bY_b.

The purification scheme described in this chapter employed a combination of S-hexylglutathione affinity chromatography, carboxymethyl-cellulose cation exchange chromatography and chromatofocusing.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals

All chemicals used were of analytical grade (see Chapter 2, Section 2.2.1.). Cumene hydroperoxide (80% in cumene) was from MERCK-Schuchardt, Schuchardt, Munchen. Alpha-chymotrytsin was obtained from Miles Laboratories, GSH and NADPH from BDH Chemicals Ltd, and ethacrynic acid and glutathione reductase were from Sigma Chemical Company.

4.2.2. Enzyme Assays

GSH S-T activity was measured with CDNB, 1,2-dichloro-4-nitrobenzene (ϵ = 5000 $M^{-1}.cm^{-1}$ at 345 nm), trans-4-phenyl-3-buten-2-one (ϵ = -24800 $M^{-1}.cm^{-1}$ at 290 nm) and ethacrynic acid (ϵ = 5000 $M^{-1}.cm^{-1}$ at 270 nm) according to published procedures (Habig et al, 1974b; and Chapter 2, Section 2.2.3.). Glutathione peroxidase activity was measured using a coupled assay with glutathione reductase, as described by Prohaska and Ganther (1977) (see Chapter 6, Section 6.2.1.2.) with 0.1 mM cumene hydroperoxide dissolved in 10% (v/v) ethanol. Activity was calculated as µmoles NADPH oxidized per minute. All kinetic measurements were made at 25^{o}C. Protein concentrations were measured as previously described (Chapter 2, Section 2.2.4.). Specific activities were expressed in units of µmol/min/mg at 25^{o}C.

4.2.3. Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed as previously described (Chapter 2, Section 2.2.5.).

4.2.4. Gel Isoelectric Focusing

Analytical thin-layer gel elecrofocusing with polyacrylamide gels in the pH range 3.5-9.5 was performed with commercially available gels (LKB Ampholine^{R} PAGplates). The method used was that prescribed by the manufacturer. The gel was placed on the cooling plate of the LKB Multiphor apparatus with paraffin as an insulator between the gel and the apparatus. The electrode strips were evenly soaked with the appropriate electrode solutions (anode - 1 M H_3PO_4; cathode - 1 M NaOH) and applied to the gel surface. Dry sample application pieces were applied to the surface of the gel (3 cm from the cathode) using the template screen printed on the cooling plate as a guide. Samples (15-20 µl, concn. 0.5-2 mg/ml) were applied to the sample pieces with a micropipette. The electrode holder was placed on the Multiphor unit and the electrodes were aligned with the centre of the electrode strips. Finally, the safety lid was placed in position. The power supply was connected and focusing was performed for 1.5 hours (power settings were : 1500 V; 50 mA; 30 W). After 45 min the sample application pieces were removed and the experiment was then continued for a further 45 min. Following focusing, the plate was immediately removed and fixed for 1 hour in a solution comprising 57.5 g trichloroacetic acid, 17.25 g sulphosalicylic acid and 500 ml distilled water. This serves to precipitate the proteins and allows the ampholines to diffuse out of the gel. Following precipitation, the plate was briefly washed (5 min) in destaining solution comprising 25% v/v ethanol and 8% v/v acetic acid. Gels were then stained for 20 min in staining solution comprising 0.46 g Coomassie Blue R 250 in 400 ml destaining solution. Destaining was carried out with several changes of destaining solution until the background was clear. Protein standards used were trypsinogen (pI = 9.30), lentil lectin-basic band (pI = 8.65), lentil lectin-middle band (pI = 8.45), lentil lectin-acidic band (pI = 8.15), myoglobin-basic band (pI = 7.35), myoglobin-acidic band (pI = 6.85), human carbonic anhydrase B (pI = 5.85), beta-lactoglobulin A (pI = 5.20), soybean trypsin inhibitor (pI = 4.55) and amyloglucosidase (pI = 3.50). The pI of unknown proteins were determined by placing the gel over a piece of graph paper so that the origin of the grid was lined up with the cathode end of the gel. The distance from the cathode of each protein pI marker was determined and the known pI value of each marker protein versus its distance from the cathode was plotted and the points connected to obtain a pH gradient calibration curve. The unknown pI of a protein could then determined by measuring the distance migrated from the cathode and reading off the pI from the pH calibration curve.

4.2.5. Cross-Linking

The purified transferase was cross-linked using a modification of the procedure described by Boyer and Kenney (1985). To favour cross-linking within protomers rather than between protomers, low protein concentrations were used (Davies and Stark, 1970). The transferase (12 µg/50 µl) was added to 100 µl of 100 mM sodium pyrophosphate buffer (pH 8.5) containing 10 mM dimethylpimelimidate dihydrochloride (Pierce Chemical Company, Rockford, IL., USA). The mixture was incubated at 30°C for 5 hours and then 2-mercaptoethanol, glycerol and bromophenol blue were added to final concentrations of 1, 2, 10 and 0.002%, respectively, and heated to 95°C for 10 min. The cross-linked protein was then subjected to SDS-polyacrylamide gel electrophoresis.

4.2.6. Peptide Mapping

This was carried out by the method of Cleveland et al (1977) as described by Hayes et al (1979) for GSH S-T Y_aY_a and Y_aY_c. Purified isozymes (25 µg/80 µl) were heated to 95°C for 2 min in the presence of 0.2% SDS and 1 mM-EDTA. These mixtures were cooled and proteolytic digestion was carried out at 37°C by the addition of various amounts of alpha-chymotrypsin (10 µl, containing 7, 0.7, 0.07, 0.007 or 0.0007 µg of protein) to the purified isozymes. After 45 min, 2-mercaptoethanol, SDS, glycerol and bromophenol blue were added to final concentrations of 1, 2, 10 and 0.002% respectively and the digestions were terminated by heating to 95°C for 10 min. The digests were analysed by SDS-polyacrylamide gel electrophoresis. Alpha-chymotrypsin in the highest concentration used for digestion, served as control.

4.2.7. Amino Acid Analysis

The amino acid analysis was carried out using high performance liquid chromatography (HPLC). Purified protein (approximately 50 µg) was freeze dried. Acid hydrolysis of the protein was performed at 100°C in 50 µl 6M HCl containing 1.3 µl 1% (w/v) phenol, for 25 hours. Homocysteic acid was used as an internal standard. The amino acids were subjected to pre-column derivatization with o-phthalaldehyde (Hill et al, 1979) followed by analysis using a reverse-phase C18 column (Waters) on a Waters (WISP 710B) HPLC. Pre-column derivatization produces derivatives which are less polar than the free amino acids (Turnell and Cooper, 1982) which makes the method ideally suited to amino acid analysis by reversed-phase chromatography. Peaks were integrated by an on-line computer and results are expressed as residues/mol.

4.2.8. Monoclonal Antibody Production

Coomassie blue-stained bands of purified GSH S-T, separated by SDS-polyacrylamide gel electrophoresis (total protein approximately 16 µg), were excised, emulsified with Freund's complete adjuvant and injected i.p. into female BALB/c mice. Booster innoculations of antigen emulsified with Freund's incomplete adjuvant were given at fortnightly intervals. Three days after the last injection (antigen in 0.01 M sodium phosphate (pH 7.4)/0.15 M NaCl), immune spleen cells were obtained from the mice and fused with mouse myeloma SP2/OAg14 cells in the presence of polyethylene glycol (PEG 4000) according to a standard procedure (Kohler and Milstein, 1975). Then approximately 1 x 10^6 cells per well were plated in 24-well tissue culture plates (Cel-Cult, Sterilin Ltd., Feltham, England). The cells were grown in RPMI 1640 (GIBCO, Scotland) containing 5% human umbilical cord serum, 10^{-4} M hypoxanthine, 1.6 x 10^{-5} M thymidine and 4 x 10^{-7} M aminopterin (Kennett et al, 1978). After 10 to 14 days, the supernatants from individual cell colonies which survived selection by aminopterin were screened for antigen-binding activity by an enzyme-linked immunosorbent assay as described below (Section 4.2.9.). Positive hybridoma cultures were cloned twice by limiting dilution without a feeder layer to give 1-2 cells per well. Finally, the supernatants from cultures still giving a positive reaction to antigen were tested for specificity by "Western Blot" as previously described (Chapter 2, Section 2.2.9.) with slight modifications. After the nitrocellulose sheet had been blocked with 3% bovine serum albumin, the paper was placed into 5 ml of positive cell culture supernatant instead of the specific antiserum. Thereafter the standard blotting method was followed.

4.2.9. Enzyme-Linked Immunosorbent Assay (ELISA)

Samples of GSH S-T antigen were diluted in PBS-9 (150 mM NaCl, 10 mM Na_2HPO_4 (pH 9.0)) and approximately 0.2 µg of antigen was adsorbed to the wells of 96-well activated polyvinyl chloride (PVC) plates (Falcon 3911 Micro Test flexible assay plates) overnight at 4°C. The wells were then blocked with 1% fetal calf serum in PBS-9 for 15-30 mins at room temperature. Blocking solution was decanted from the wells and 0.05 ml supernatant from hybridoma cells was added to the wells of the PVC plates and incubated for 1 hour at room temperature. The wells were then washed ten times with "TST" solution comprising 0.1 M NaCl, 0.05% Tween 20 in 0.05 M Tris-HCl (pH 8.0). Antibodies bound to the GSH S-T were detected by further incubation for 30 min with 0.05 ml of peroxidase-labeled goat anti-mouse immunoglobulins (Cooper Biomedical, Inc., Malvern, PA, USA) diluted 1:300 in a solution comprising 0.1% bovine serum albumin, 0.5 M NaCl, 0.01 M Na_2HPO_4, 0.5% Triton-X-100. The wells were again washed with TST, and stained by the addition of 0.01 ml 2,2'azino-di(3-ethyl-benzthiazoline)-sulfonic acid (ABTS) (final concentration 0.4 mM in 0.1 M sodium citrate buffer (pH 4.0)) to each well. Immediately before use 0.1 ml hydrogen peroxide (30%) was added to 10 ml of the ABTS solution. The colour reaction was allowed to develop for 15 mins and the absorbance of each well was read at 414 nm on an ELISA reader (Titertek Multiskan^R MCC/340, Flow Lab., Ltd, Ayrshire, Scotland, UK). All plates included a known positive control, a negative control (no antigen adsorbed to the well) and a non-immune control.

4.2.10. GSH S-Transferase Purification

The cytosol fraction from the livers of rat fetuses which were 17 or 18 days old, was obtained as previously described (Chapter 3, Section 3.2.5.). A 50-ml aliquot of the cytosol fraction was chromatographed on a Sephadex G-25 (Pharmacia) column (3.2 x 60 cm) previously equilibrated with 0.01 M Tris-HCl buffer, pH 7.80 (buffer A). Fractions exhibiting GSH S-T activity with CDNB as substrate were pooled and applied to an affinity column (1.6 x 15 cm) of S-hexylglutathione linked to epoxy-activated Sepharose 6B, equilibrated with buffer A. The column was washed with 200 ml of 0.01 M Tris-HCl (pH 7.8)/0.2 M NaCl after which the GSH S-T were eluted with 5 mM S-hexylglutathione in the same buffer. Fractions showing absorption at 280 nm were pooled and applied to a Sephadex G-25 column (1.6 x 60 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 6.0) (buffer B). Fractions exhibiting GSH S-T activity with CDNB were pooled and applied to a carboxymethyl-cellulose (CM52) cation exchange column (1.6 x 10 cm) equilibrated with buffer B. The column was washed with 30 ml of the above buffer and then developed with a linear salt gradient of 0-75 mM KCl in 0.01 M sodium phosphate buffer (pH 6.0) in a total volume of 720 ml of buffer. Fractions of 3 ml were collected and assayed for transferase activity. The peak fractions of transferase activity eluted at approximately 60 mM KCl (fractions 205-225) were pooled, concentrated with stirring over an Amicon PM 10 membrane and applied to a Sephadex G-25 column (1.6 x 60 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.0). Fractions exhibiting GSH S-T activity with CDNB were pooled, concentrated, and applied to a PBE118 chromatofocusing column (1.0 x 30 cm) equilibrated with 25 mM Triethylamine-HCl buffer (pH 11.0). Elution was carried out with 500 ml of Pharmalyte (pH 8-10.5), diluted 1:80, and adjusted with HCl to pH 8.0. Fractions of 2 ml were collected and assayed for transferase activity. Those fractions containing the purified enzyme, as evidenced by SDS-polyacrylamide gel electrophoresis, were pooled and subjected to either S-hexylglutathione affinity chromatography or Sephadex G-75 chromatography (1.6 x 90 cm column) to remove the ampholines. The purified enzyme was frozen at -70^{o}C until used.

4.2.11. Preparation of Adult Rat Liver GSH S-Transferases

The adult rat liver glutathione S-transferases used for comparative purposes were purified as described previously (Chapter 2, Section 2.2.11.).

4.3. RESULTS

Table 4.1 summarizes the purification. Following affinity chromatography of the fetal liver cytosol, six major GSH S-T activity peaks were resolvable by CM-cellulose chromatography (Fig. 4.1). They were present in fractions 15, 80, 100, 130, 215 and 235 and were referred to as peaks I-VI. Peak I eluted before the application of a salt gradient and comprises acidic isoenzymes which do not bind to the CM-cellulose column. On SDS-polyacrylamide gel electrophoresis peaks II and III comprised subunits of the Y_b/Y_b' type and peak IV comprised subunits of the Y_a, Y_b/Y_b' and Y_c type. The minor peak represented by fraction 185 also comprised subunits of the Y_b/Y_b' type. The peak of activity eluting at approximately 60 mM KC1 (peak V) comprised two subunits. One of these subunits was identified as subunit Y_c from its electrophoretic mobility while the other subunit was identical with the fetal subunit described in Chapter 3. The peak of activity represented by fraction 235 (peak VI) comprised subunit Y_c with traces of the fetal subunit, probably resulting from contamination by peak V.

Peak V (fractions 205-225) was further purified by chromatofocusing to separate possible isozymes according to their isoelectric points. This yielded two peaks of GSH S-T activity (Fig. 4.2). The minor peak represented by fraction 51 eluted from the column at a pH of approximately 9.75 and on SDS-polyacrylamide gel electrophoresis comprised one band (M_r=28 000). This identified it as the homodimer GSH S-T Y_cY_c. The major peak eluting at a pH of approximately 9.3 - 9.6 and represented by fraction 66 comprised two nonidentical

Table 4.1. **SUMMARY OF THE PURIFICATION OF THE FETAL RAT LIVER GLUTATHIONE S-TRANSFERASE ISOENZYME FROM FETAL RAT LIVER CYTOSOL**

Purification Step	Total Protein (mg)	Total Activity* (µmol/min)	Specific Activity (µmol/min/mg)	Yield (%)
Cytosol	796	271	0.34	100
Sephadex G-25	554	255	0.46	94
Affinity chromatography + Sephadex G-25	7.5	181	24.1	67
CM-cellulose (fractions 205-225)	0.95	9.6	10.1	3.5
Sephadex G-25	0.86	8.4	9.8	3.1
PBE118 column (fractions 60-80) + Sephadex G-75	0.75	6.2	8.3	2.3

*Glutathione S-transferase activity was measured with CDNB according to Habig et al (1974b) (Section 2.2.3.)

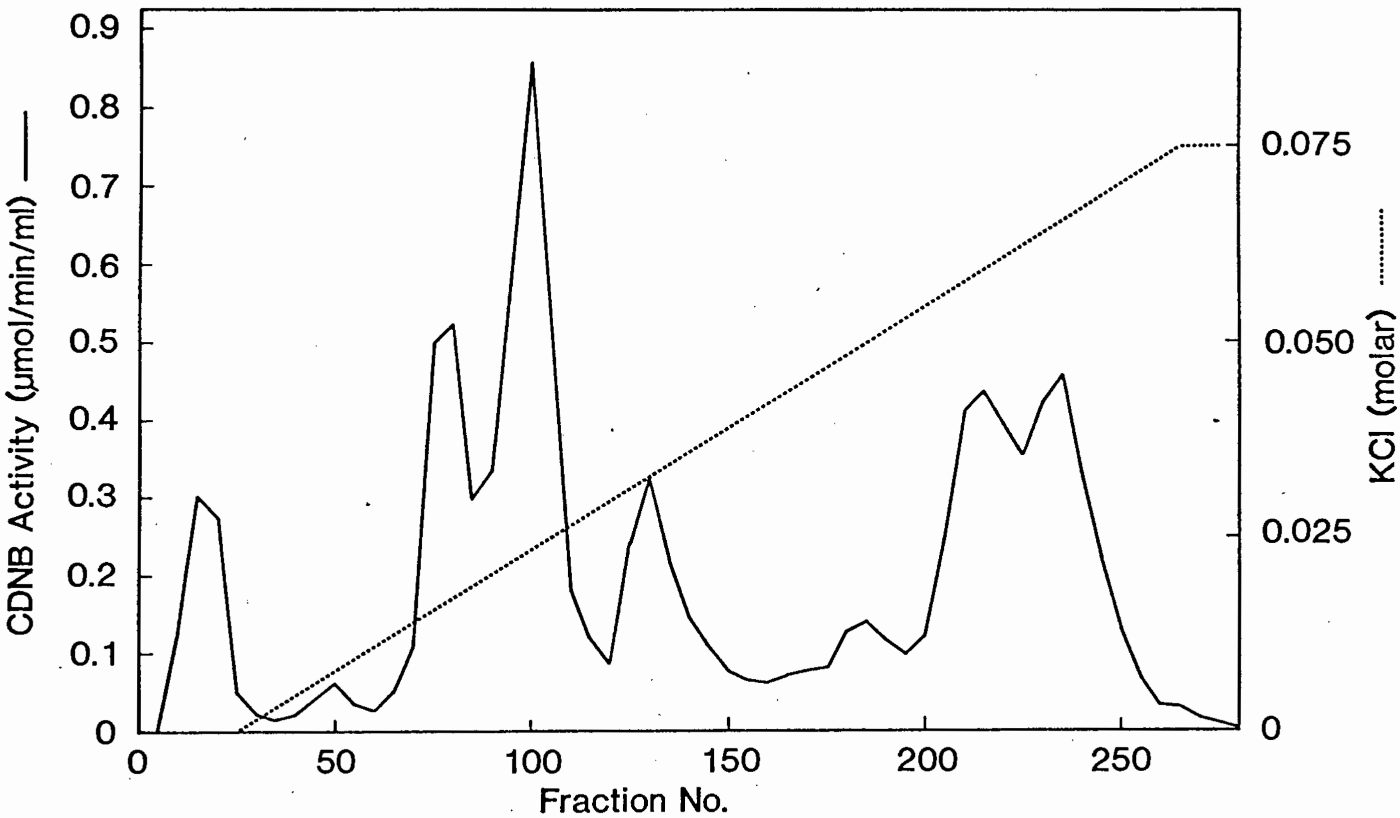


Fig. 4.1. **Resolution of fetal rat liver GSH S-T isozymes by carboxymethyl-cellulose column chromatography. The activity was measured with CDNB and glutathione as substrates.**

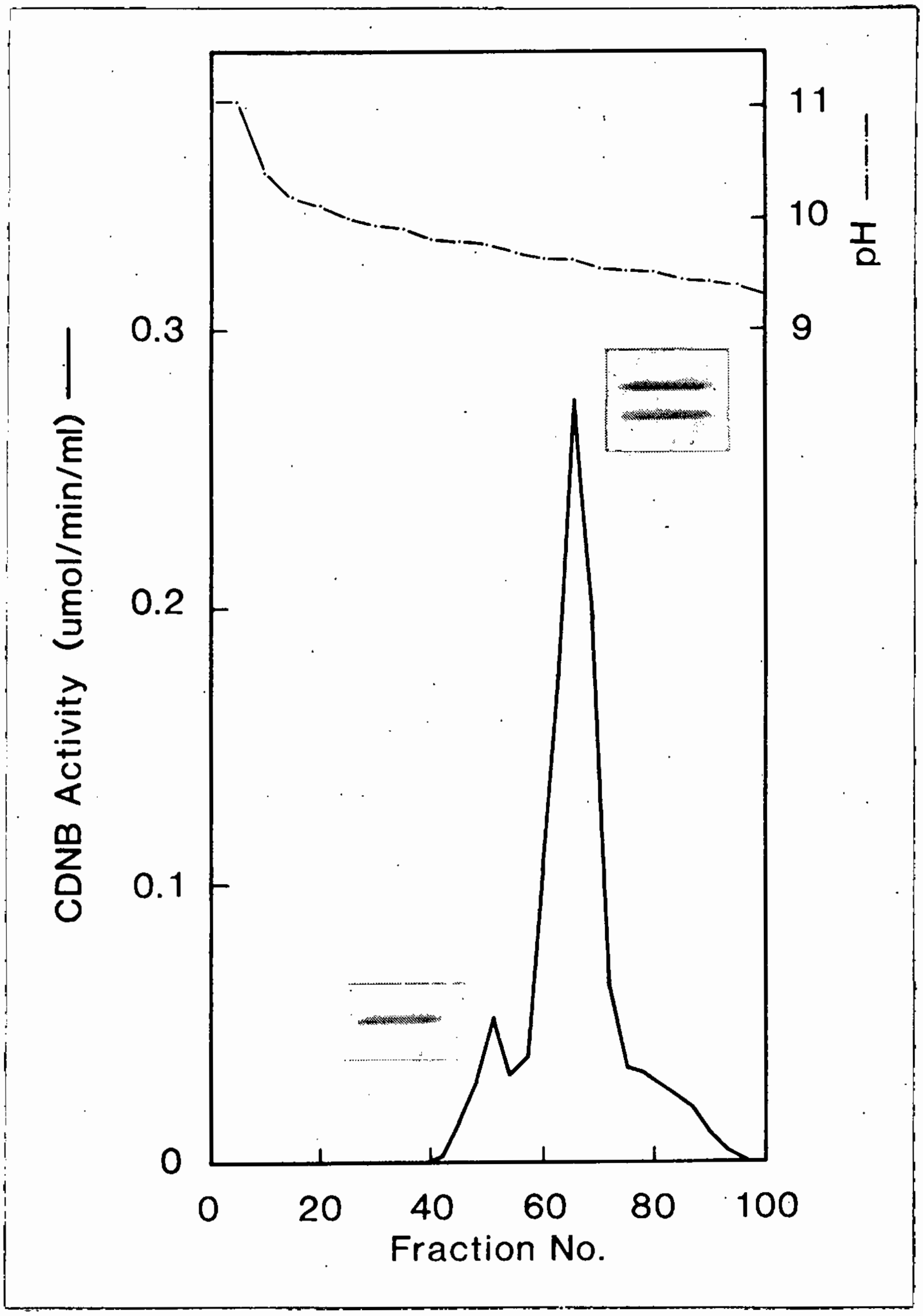


Fig. 4.2. Chromatofocusing of peak V (fractions 205-225) obtained by carboxymethyl-cellulose column chromatography. The insets show the subunit composition of fractions 51 and 66 on SDS-polyacrylamide gel electrophoresis.

subunits, namely, subunit Y_c and the fetal subunit (M_r=25 500) which is now referred to as subunit 'Y_{fetus}'.

Fig. 4.3 shows the subunit composition of this novel fetal rat liver GSH S-T isozyme (lane 3) with adult rat liver GSH S-T Y_aY_c (lane 4) and $Y_bY_{b'}$ (lane 5) as references. Lane 2 shows the subunit composition of the GSH S-T derived from fetal rat livers by affinity chromatography.

Densitometric scanning (Beckman Appraise^{TM} Densitometer) of the Coomassie blue-stained bands obtained from SDS-polyacrylamide gel electrophoresis indicated that these two subunits were present in approximately equal amounts (Fig. 4.4) providing further evidence that the enzyme was pure. The dimeric nature of the enzyme was studied by cross-linking the subunits with dimethylpimelimidate and then subjecting the cross-linked subunits to electrophoresis. This yielded a major band corresponding in molecular weight to a combination of the two subunits. A minor, less well-defined band with a M_r value of approximately 47 500 and therefore not related to a dimer of either subunit was also detectable. This band is probably the result of protein breakdown induced by the cross-linking agent and the conditions under which the cross-linking procedure was performed.

Peptide mapping by limited proteolysis of GSH S-T Y_cY_{fetus} and Y_cY_c revealed two major digestion products of the Y_cY_{fetus} isozyme which were absent from Y_cY_c (Fig. 4.5). These products had M_r values of approximately 24 500 and 22 900. Several digestion products with M_r values of approximately 25 300 and 15 600 to 18 800 were common to both isozymes. These peptide maps are reproducible and clearly show that GSH S-T Y_cY_{fetus} is distinct from the Y_cY_c isozyme and is not

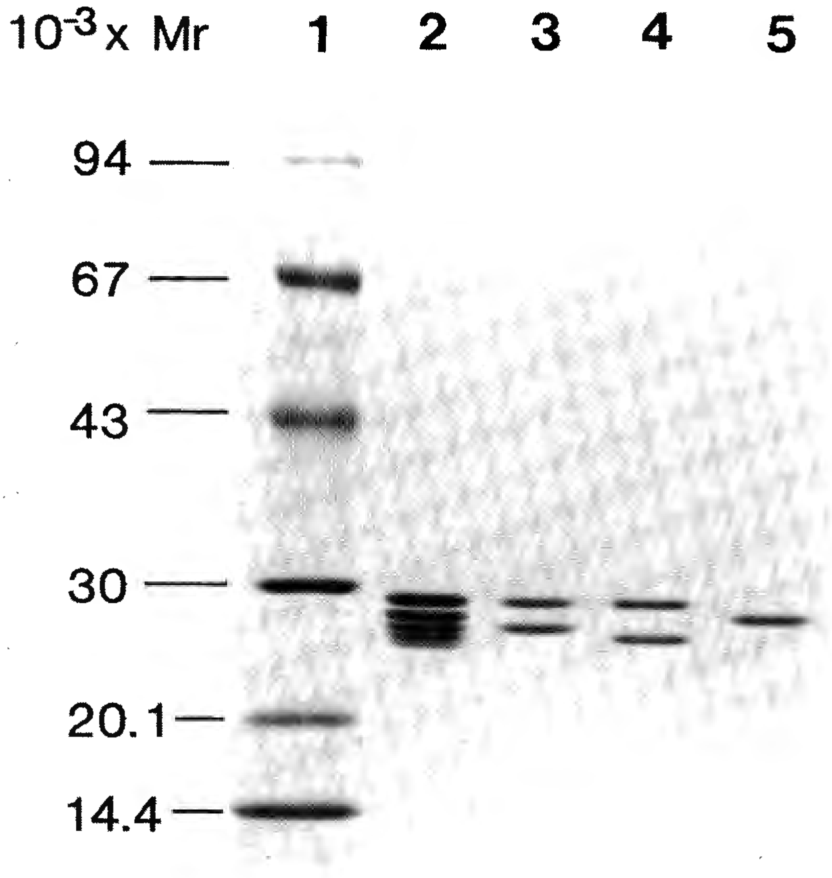
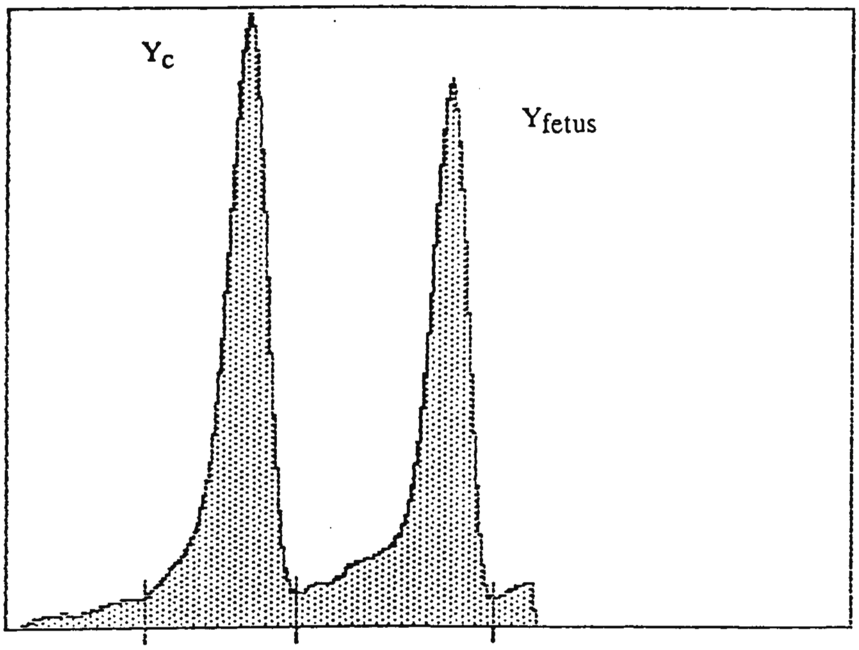


Fig. 4.3. SDS-polyacrylamide gel electrophoresis showing the subunit composition of the novel fetal rat liver GSH S-T isozyme (lane 3) with adult rat liver GSH S-T Y_aY_c (lane 4) and $Y_bY_{b'}$ (lane 5) as references. Lane 2 shows the subunit composition of the GSH S-T derived from fetal rat livers by affinity chromatography and lane 1 molecular weight markers.



Fraction	Relative %
Y_c	51
Y_{fetus}	49

Fig. 4.4. Densitometric scanning of the Coomassie blue-stained bands obtained from SDS-polyacrylamide gel electrophoresis of purified GSH S-T Y_cY_{fetus}.

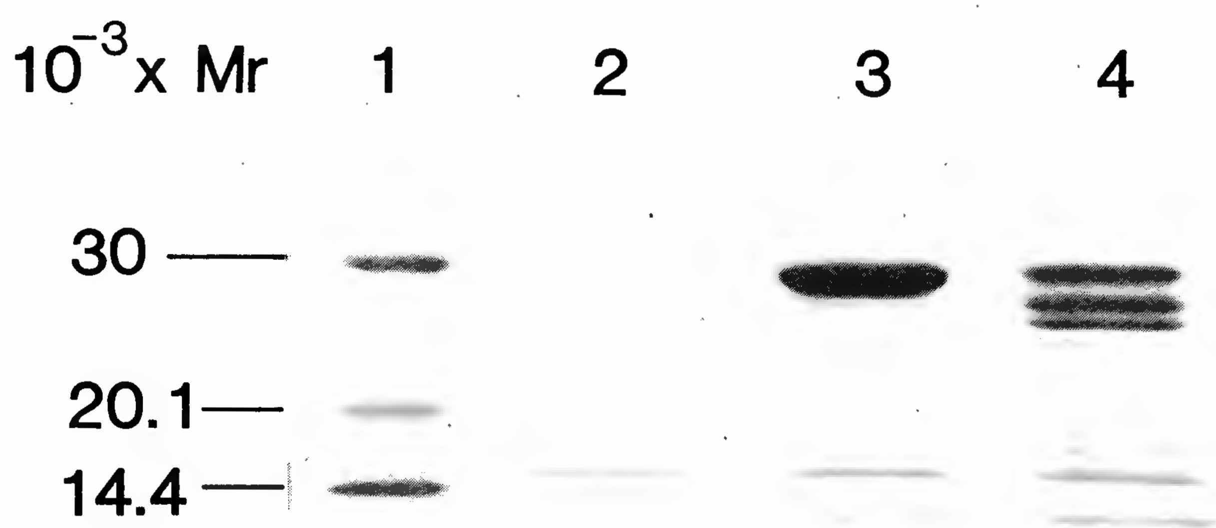


Fig. 4.5. Peptide mapping of GSH S-T Y_cY_{fetus} and Y_cY_c by limited proteolysis. Purified isozymes (25 µg) were digested with 7 µg of alpha-chymotrypsin as described under Materials and Methods (Section 4.2.6.). The digests were then subjected to SDS-polyacrylamide gel electrophoresis. The samples applied were: (1) molecular weight markers; (2) alpha-chymotrypsin (7 µg); (3) Y_cY_c isozyme (25 µg) and chymotrypsin (7 µg); (4) Y_cY_{fetus} isozyme (25 µg) and chymotrypsin (7 µg).

generated by proteolysis of Y_cY_c.

The amino acid composition of the fetal isozyme is given in Table 4.2. The values obtained were similar to those reported previously for the adult rat liver transferases (Jakoby, 1978; Mannervik, 1985a) with the exception of arginine and lysine which gave higher values.

Various substrates were used to measure enzymatic activity. A summary of the substrate specificities of the fetal isozyme compared with those of some of the adult rat liver GSH S-transferases and which do not differ significantly from those obtained by others under similar conditions (Boyer et al, 1983) is given in Table 4.3. The activity towards the standard and most highly reactive adult substrate, CDNB, was relatively low. However, with the model organic substrate for glutathione peroxidase activity, cumene hydroperoxide, the fetal isozyme had one of the highest activities yet described for purified rat liver GSH S-T.

Table 4.2. AMINO ACID COMPOSITION OF THE NOVEL FETAL RAT LIVER GLUTATHIONE S-TRANSFERASE ISOENZYME (Y_cY_{fetus})

Amino acid	Amino acid composition (residues/mole)
Asp	31,2
Glu	35.0
Cys	1.6
Ser	10.9
Gln	9.3
His	1.1
Thr + Gly*	43.2
Ala	25.2
Tyr	12.6
Arg	55.8
Val	18.0
Met	0.6
Ile	11.5
Leu	40.5
Phe	12.6
Lys	57.4

* Could not be separated individually on HPLC Calculations were based on the molecular weight of Thr.

Table 4.3. SUBSTRATE SPECIFICITIES OF THE FETAL RAT LIVER GLUTATHIONE S-TRANSFERASE ISOENZYME (Y_cY_{fetus}) COMPARED WITH SOME OF THE ADULT RAT LIVER GSH S-TRANSFERASES

Values for isoenzyme activity are given in µmol/min per mg.

SUBSTRATE	ISOENZYME						
	Y_aY_a	Y_aY_c	Y_cY_c	Y_bY_b	$Y_bY_{b'}$	$Y_{b'}Y_{b'}$	Y_cY_{fetus}
1-Chloro-2,4-dinitrobenzene	31.4	25.2	14.5	30.2	20.1	12.2	8.3
1,2-Dichloro-4-nitrobenzene	0.04	0.04	0.05	5.3	3.2	0.15	0.9
trans-4-Phenyl-3-buten-2-one	0	0	0	0	0.36	1.2	0
Ethacrynic acid	0.1	0.52	1.2	0.08	0.19	0.62	0.13
Cumene hydroperoxide	2.3	4.1	3.8	0.2	0.3	0.4	7.9

4.4. DISCUSSION

This chapter has described the purification and some properties of a novel fetal rat liver GSH S-T isozyme. This isozyme is heterodimeric comprising subunits Y_c and 'Y_{fetus}' and hence referred to as GSH S-transferase Y_cY_{fetus}. The dimeric nature of the isozyme was confirmed by cross-linking of the subunits with dimethylpimelimidate as well as by densitometric scanning of the two subunits on SDS-polyacrylamide gel electrophoresis.

The fetal isozyme is a basic protein which eluted from a chromatofocusing column at a pH of approximately 9.3 to 9.6. Analytical thin layer gel electrofocusing with polyacrylamide gels in the pH range 3.5 - 9.5 yielded a single band with an isoelectric point of approximately 8.65 (Fig. 4.6), this agreed closely with the pI of approximately 8.50 previously predicted as an isoelectric point for the isozyme(s) comprising the fetal subunit (Chapter 3). Densitometric measurements (Chapter 3) have suggested that the fetal subunit and hence GSH S-T Y_cY_{fetus} may account for as much as 26% of the GSH S-T in fetal rat livers.

It was previously shown with Western blotting, that the fetal subunit reacts with antibodies raised against GSH S-T Y_aY_c and Y_cY_c (Chapter 3) although immunodiffusion of adult and fetal GSH S-T against these two antisera suggested that this identity was incomplete. The purified fetal isozyme was studied by immunodiffusion using antibodies raised against GSH S-T Y_aY_c, Y_cY_c, Y_bY_b, $Y_bY_{b'}$ and $Y_{b'}Y_{b'}$. Precipitates were obtained with the antisera to transferases Y_aY_c and Y_cY_c but not with those to transferases Y_bY_b, $Y_bY_{b'}$ or $Y_{b'}Y_{b'}$. This

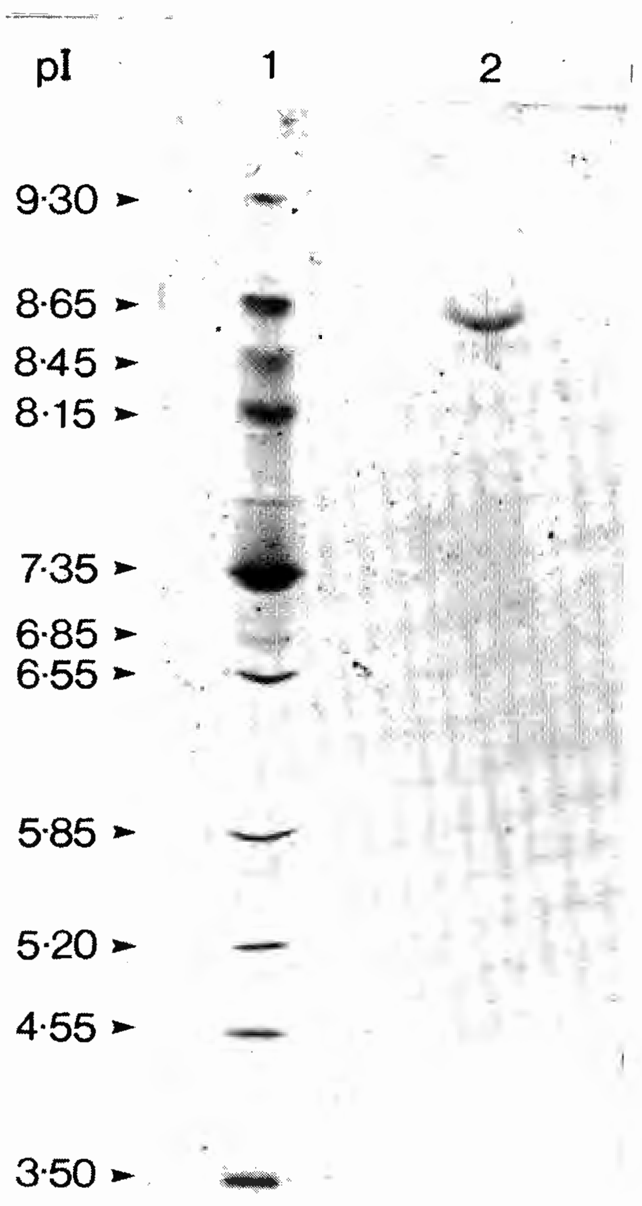


Fig. 4.6. **Analytical thin layer gel isoelectric focusing of purified GSH S-T Y_cY_{fetus} (lane 2). Lane 1 shows standard pI markers (see Section 4.2.4. for details).**

confirmed the Western blot results obtained in Chapter 3 in which the fetal subunit was found to react with antibodies raised against GSH S-T Y_aY_c and Y_cY_c but not GSH S-T Y_aY_a, Y_bY_b, $Y_bY_{b'}$ or $Y_{b'}Y_{b'}$. These results indicate that the fetal isoenzyme bears antigens shared with transferases Y_aY_c and Y_cY_c i.e., related the Y_c subunit.

The antigenic similarity between subunits Y_c and Y_{fetus} was illustrated by attempts to develop a monoclonal antibody to the fetal subunit. The purified isoenzyme was subjected to SDS-polyacrylamide gel electrophoresis and the Coomassie blue-stained fetal subunit was then excised, homogenized, and injected into mice (Section 4.2.8.). Despite extensive attempts at obtaining a monospecific antibody, during which 14 positive clones from two different mice were obtained, all positive cell supernatants showed partial recognition of the Y_c subunit on Western blot (Fig. 4.7.). This could not be accounted for by non-specific binding to the Y_c subunit as immunological cross-reactivity was not evident with the other adult subunits on Western blot. It is possible that small quantities of the Y_c subunit were 'dragged' with the fetal subunit on SDS-polyacrylamide gel electrophoresis accounting for the immunological cross-reactivity. However, previous experience with the quantities of antigen required to elicit an immune response suggest that this was unlikely. Experiments were also conducted in which the purified fetal isoenzyme was subjected to limited proteolysis with alpha-chymotrypsin followed by Western blot with rabbit anti-Y_cY_c antiserum. The results showed that the fetal subunit could be digested to peptides which did not bear antigenic similarity with the Y_c subunit (Fig. 4.8) and may provide a means of developing an antibody which is specific for the fetal subunit.

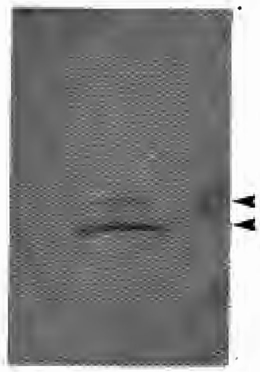


Fig. 4.7. Western blot of GSH S-T Y_cY_{fetus} using the supernatants from positive cell clones (see Section 4.2.8. for details).

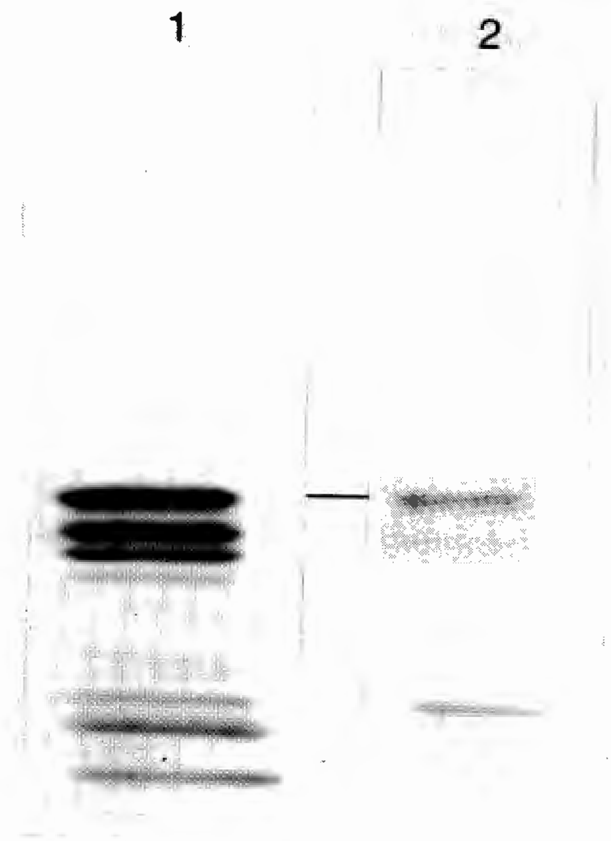


Fig. 4.8. Limited proteolysis of GSH S-T Y_cY_{fetus} with alpha-chymotrypsin followed by Western blot with rabbit anti-Y_cY_c antiserum (lane 2). Lane 1 shows the unblotted digest.

Over a dozen purifications of GSH S-T Y_cY_{fetus} failed to provide evidence for the existance of a homodimer comprising the fetal subunit. It thus differs from the four major cationic subunits which form homo- and hetero- dimers, although deviation from their behaviour has been shown for other rat GSH S-T, example, GSH S-T Y_pY_p and GSH S-T 8-8, both of which only appear to exist as homodimers (Chapter 1, Section 1.4.1.).

Little is currently known about the GSH S-T isozyme composition of fetal rat livers. Ontogenic studies have shown that the cytosolic activities of the hepatic GSH S-T in the fetal and perinatal period are relatively low, rising to adult levels 4-5 weeks after birth (Chapter 1, Section 1.8.1.). GSH S-T Y_cY_{fetus} utilizes a range of substrates. With cumene hydroperoxide as the substrate it posesses higher GSH peroxidase activity than is usual for hepatic GSH S-T.

The GSH peroxidases serve a critical function in inhibiting lipid peroxidative processes in biological membranes. Targets for lipid peroxidation are the subcellular membranes rich in unsaturated fatty acids and peroxidation results in loss of both structural integrity and function in the affected organelles. In addition, products of lipid peroxides, such as aldehydes, migrate from their site of production causing damage at distant loci (Chapter 1, Section 1.11.4.). Compared with adult rat livers, high levels of spontaneous lipid peroxidation have been observed in fetal rat liver homogenates obtained from 19-day fetuses (Utsumi et al, 1977). In vivo lipid peroxidation measured by expired gases also seems to be higher in the case of newborn infants and rabbits compared with adults (Wispe et al, 1985). Similar high levels of lipid peroxidation have been observed in human blood during the neonatal period (McCarthy et al, 1984). Superoxide dismutase, regarded as a major defence against oxygen toxicity and lipid peroxidation (Bus and Gibson, 1979; Yoshioka et al, 1977) has been shown to be present in low levels in fetal rat liver homogenates (Utsumi et al, 1977) and in the hepatic microsomes of newborn rats (Devasagayam, 1986). It is therefore attractive to suggest that GSH S-T Y_cY_{fetus} may serve a specific function in the fetal liver in protecting the fetus against the possible teratogenic effects of organic peroxides.

CHAPTER 5

INHIBITION OF RAT LIVER GLUTATHIONE S-TRANSFERASE ISOENZYMES BY ACROLEIN

5.1. INTRODUCTION

Acrolein (prop-2-enal) is a toxic aldehyde used in the manufacture of colloidal forms of metals, making of plastics, gas warfare, organic synthesis, etc. It is found in the vapor phase of cigarette smoke (Newsome et al, 1965) and in the engine exhaust of automobiles (Tonimoto and Uehara, 1975). Acrolein is a metabolite of the anticancer drug cyclophosphamide and Slott and Hales (1985) have recently shown that it is acrolein which is the teratogenic component of cyclophosphamide.

The GSH S-T appear to be responsible for the initial step in the metabolic conversion of acrolein to a mercapturic acid (3-hydroxypropylmercapturic acid) which can be isolated from the urine of rats following the subcutaneous administration of acrolein (Kaye, 1973). Fig. 5.1 shows three routes for the biotransformation of acrolein in the rat. Inhibition of the GSH S-T by their non-substrate and substrate ligands including sulphobromophthalein, epoxides, benzyl chloride, etc. have been reported (Chapter 1, Section 1.2.2.1.). Chapter 4 described the identification of a novel fetal rat liver GSH S-T isoenzyme. I therefore thought it would be of interest to look at the in vitro effect of

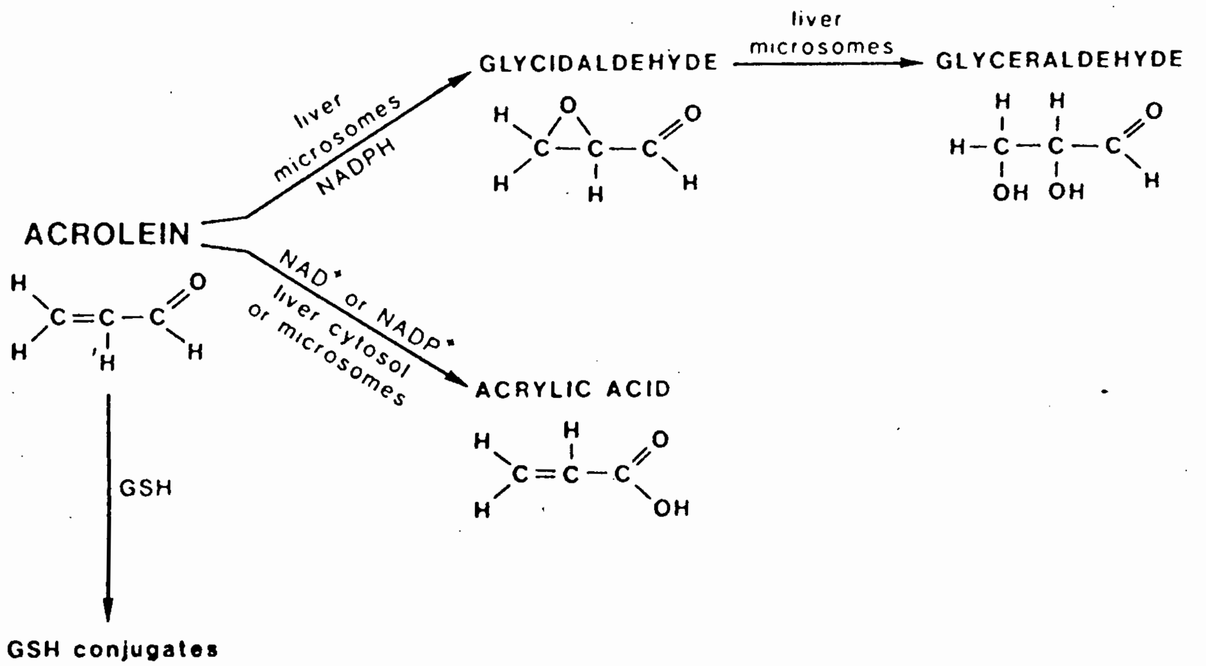


Fig. 5.1. Three metabolic routes for the biotransformation of acrolein in the rat.

the teratogen, acrolein, on this fetal isoenzyme and to compare it with some of the adult rat liver GSH S-T isoenzymes.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals

All chemicals used were of analytical grade (see Chapter 2, Section 2.2.1.). Acrolein was from the Sigma Chemical Company.

5.2.2. Purification of Rat Liver GSH S-Transferases

The adult rat liver GSH S-T were purified as described previously (Chapter 2, Section 2.2.11.). GSH S-T Y_cY_{fetus} was purified as described in Chapter 4 (Section 4.2.10.).

5.2.3. Enzyme Inhibition Studies

The inhibition studies were performed with CDNB as enzyme substrate. Various concentrations of acrolein were prepared in ethanol (95%). Enzyme solution (10 µl) was pre-incubated in 0.1 M phosphate buffer (pH 6.50) for 5 min at 25°C with 10 µl of inhibitor solution at various concentrations. After pre-incubation GSH (1 mM) and CDNB (1 mM) were added to the incubation mixture and GSH S-T activity was determined according to the method of Habig et al (1974b) (Chapter 2, Section 2.2.3.). A blank contained all of the above with enzyme source replaced by buffer identical to that of the protein solution. The concentration of inhibitor resulting in 50% inhibition of GSH S-T activity, the I_{50} value, was determined from plots of remaining activity versus inhibitor concentration. Control values were obtained by pre-incubation with an appropriate volume of solvent in the absence of inhibitor.

5.3. RESULTS AND DISCUSSION

Fig. 5.2 shows the in vitro effect of acrolein on some of the rat liver GSH S-T. The enzymes were inhibited by acrolein to varying degrees. The isoenzymes comprising the Y_b and $Y_{b'}$ subunits were the most susceptible with I_{50} values of 4.1 x 10^{-4} and 5.4 x 10^{-4} M for GSH S-T Y_bY_b and $Y_{b'}Y_{b'}$, respectively (Table 5.1). GSH S-T Y_aY_c, Y_cY_c and Y_cY_{fetus} were less susceptible to inhibition with I_{50} values of 9.3 x 10^{-4}, 8 x 10^{-4} M and 7 x 10^{-4} M, respectively. Incubation of the GSH S-T isoenzymes with 10 µl of ethanol (95%) in the absence of acrolein did not result in this loss of activity. Pre-incubation of the Y_cY_c isoenzyme with acrolein followed by overnight dialysis against 0.01 M sodium phosphate (pH 7.4)/0.1 M NaCl showed this inhibition to be reversible.

The major basic cytosolic GSH S-T of the rat liver may conveniently be divided into two groups based on structural and catalytic properties, the first comprising GSH S-T Y_aY_a, Y_aY_c and Y_cY_c, and the second comprising GSH S-T Y_bY_b, $Y_bY_{b'}$ and $Y_{b'}Y_{b'}$ (Mannervik and Jensson, 1982; Mannervik et al, 1985; and Chapter 1, Section 1.4.1.). The fetal isoenzyme Y_cY_{fetus} may be placed into the first group based on a combination of its structural, catalytic and immunological properties (Chapter 4) and the susceptibility of the fetal isoenzyme to inhibition by acrolein appears to substantiate this.

Pre-incubation of the isoenzymes with 1 mM GSH before the addition of acrolein failed to protect the enzymes from inhibition suggesting that acrolein did not inhibit enzyme activity by virtue of its binding to the GSH binding site on the enzyme.

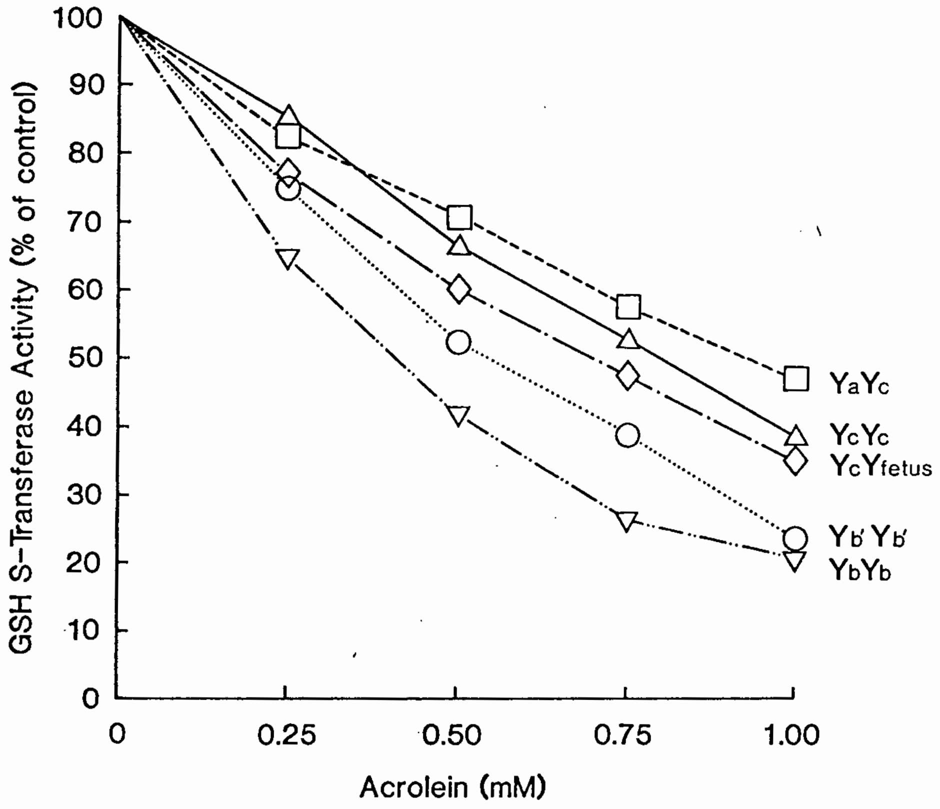


Fig. 5.2. Inhibition of rat liver GSH S-T by varying concentrations of acrolein. Enzyme activity was measured with GSH and CDNB as the electrophilic substrate.

Table 5.1. **INHIBITION OF RAT LIVER GSH S-TRANSFERASES BY ACROLEIN**

Isoenzyme	I_{50} (M)	Nature of inhibition with respect to CDNB
Y_aY_c	9.3×10^{-4}	Mixed-type noncompetitive
Y_cY_c	8.0×10^{-4}	Noncompetitive
Y_cY_{fetus}	7.0×10^{-4}	Mixed-type noncompetitive
Y_bY_b	4.1×10^{-4}	Mixed-type noncompetitive
$Y_{b'}Y_{b'}$	5.4×10^{-4}	Noncompetitive

Enzyme inhibition by acrolein was measured with 1-chloro-2,4-dinitrobenzene as electrophilic substrate as described in the text. The nature of inhibition was determined from double-reciprocal plots (see Fig. 5.3 for plots).

The inhibition of the isoenzymes in the presence of a fixed concentration of acrolein was studied over a range of concentrations of CDNB and double-reciprocal plots were drawn to determine the nature of inhibition with respect to CDNB (Fig. 5.3). Surprisingly, the results suggest that enzyme inhibition is either noncompetitive or mixed-type noncompetitive (Table 5.1) rather than competitive, as would be expected for a substrate. However, the inhibition of GSH S-T Y_bY_b by the substrate benzyl chloride, has similarly been shown to be noncompetitive in the standard 1,2-dichloro-4-nitrobenzene assay (Pabst et al, 1974).

It is therefore feasible that exposure to acrolein could result in inactivation of the GSH S-T with their subsequent failure in detoxification. The fetal liver isoenzyme Y_cY_{fetus} has one of the highest GSH peroxidase activities yet described for the purified rat liver GSH S-T and it has been suggested that it may serve a specific function in protecting fetuses against the possible teratogenic effects of organic peroxides (Chapter 4, Section 4.4.). Exposure to the known teratogen, acrolein, in various guises could therefore result in inhibition of the fetal isoenzyme and its subsequent failure to inhibit lipid peroxidation. The inhibition of the GSH S-T by acrolein will be further discussed in Chapter 6.

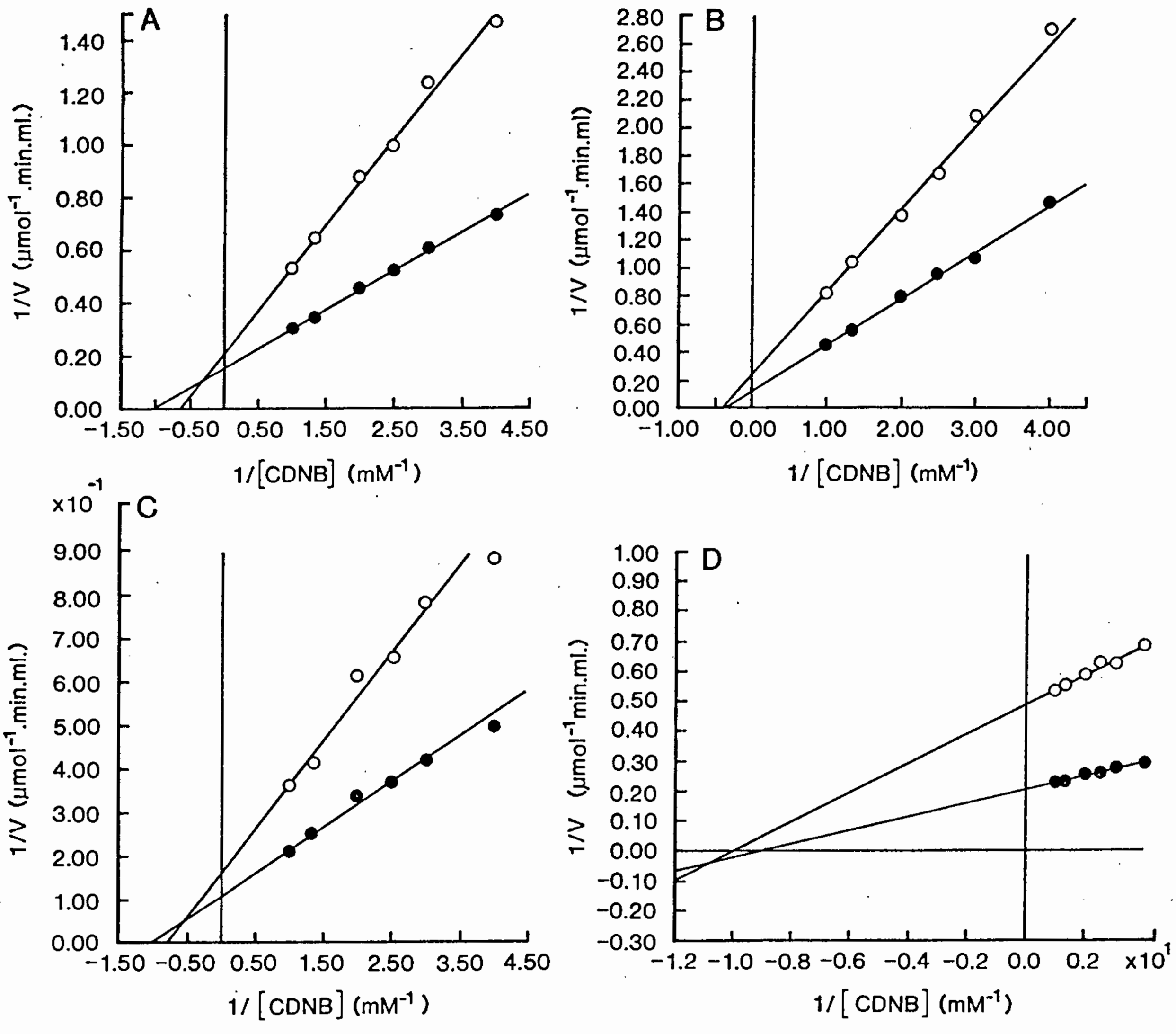


Fig. 5.3. Double-reciprocal plot showing acroleins inhibition of the fetal rat liver GSH S-T isoenzyme Y_cY_{fetus} (A), with similar plots for the adult rat liver isoenzymes Y_cY_c (B), Y_aY_c (C) and Y_bY_b (D). Activity was measured at a constant GSH concentration of 1 mM. Concentrations of CDNB were varied and acrolein concentrations were 0 mM (●) and 0.5 mM (○). Experiments were repeated in duplicate to confirm reproducibility of plots. Plots are a duplication of some data from Table 5.1.

CHAPTER 6

THE GLUTATHIONE PEROXIDASE ACTIVITY OF GLUTATHIONE S-TRANSFERASES Y_CY_{fetus} AND Y_CY_C

6.1. INTRODUCTION

The GSH S-T have been shown to bind, non-covalently, to numerous lipophilic substrate and non-substrate ligands (see Chapter 1, Section 1.2.2.1.). The binding of non-substrate ligands by the GSH S-T has established a potentially important role for these enzymes as storage and/or transport proteins. The binding of substrate and non-substrate ligands also serves to inhibit the enzyme activity of the GSH S-T towards the standard electrophilic substrates (Pabst et al, 1974; Ketley et al, 1975; Bhargava et al, 1978; Vander Jagt et al, 1982, Boyer et al, 1984) which may have important toxicological implications. Most inhibitor studies to-date have been performed using CDNB as the electrophilic substrate, and little data is available on the effects of these substrate and non-substrate ligands on the GSH peroxidase activity of the GSH S-T.

This study investigated and compared the kinetic properties of the GSH peroxidase activity of the two GSH S-T isoenzymes, Y_CY_{fetus} and Y_CY_C. The effects of the non-substrate ligand, rose bengal, as well as the substrates, sulphobromophthalein and acrolein, on the GSH peroxidase activity of these two isoenzymes was also investigated. For comparative purposes, the effects of these ligands on GSH S-T activity was also investigated. The Y_cY_c isoenzyme was chosen for comparison with the fetal isoenzyme as the Y_c subunit is associated with the high GSH peroxidase activity of the major basic rat liver GSH S-T (Chapter 1, Section 1.4.1.).

6.2. MATERIALS AND METHODS

6.2.1. Glutathione Peroxidase Assay

6.2.1.1. The Coupled Assay

Glutathione peroxidase activity was measured using a coupled assay with glutathione reductase, as described by Prohaska and Ganther (Prohaska and Ganther, 1977). The method measures the rate of GSH oxidation by the peroxide substrate as catalyzed by the glutathione peroxidase present. However, rather than measuring progressive loss of GSH, this substrate is maintained at a constant concentration by the addition of exogenous GSH reductase and NADPH, which immediately convert any oxidized GSH (GSSG) produced, to the reduced form. The rate of GSSG formation is then measured by following the decrease in absorbance of the reaction mixture at 340 nM as NADPH (ε = 6000 $M^{-1}.cm^{-1}$) is converted to NADP.

In accordance with the conditions necessary for a valid coupled assay (Segal, 1975), the following criteria need to be met for the above assay :

(i) the primary reaction must be zero-order with respect to [GSH] over the assay time and irreversible, and

(ii) the second-stage reaction must be first order with respect to [GSSG], and irreversible.

Condition (i) is easily met if only a small fraction of $[GSH]_0$ is utilized during the assay period, or if $[GSH]_0 >> K_{mGSH}$ and all cosubstrates are saturating. Irreversibility is assumed by the removal of GSSG in the second-stage reaction. Condition (ii) is met if $[GSSG]_{SS}$, the steady-state concentration of GSSG, is $<< K_{mGSSG}$ for glutathione reductase. The $[GSSG]_{SS}$ can be maintained $<< K_{mGSSG}$ by using sufficient excess of glutathione reductase. Irreversibilty can be assumed if the cosubstrate, namely, NADPH is continuoüsly removed. Under these conditions the rate of disappearance of NADPH will be constant and proportional to the GSH peroxidase present.

6.2.1.2. **Method**

The reaction mixture, in a cuvette, consisted of 0.54 ml 0.1 M potassium phosphate buffer (pH 7.0)/ 0.1 M KCl; 0.1 ml 0.03 M EDTA; 0.1 ml 0.01 M GSH; 0.1 ml 0.0011 M NADPH and 0.1 ml GSH reductase (4 ug of protein). Enzyme source (0.05 ml) was added to the above mixture and allowed to incubate for 10 min at 25°C before initiation of the reaction by the addition of 0.01 ml peroxide solution. A blank contained all of the above with enzyme source replaced by buffer identical to that of the protein solution. After a delay time of 0.3 min the change in absorbance at 340 nm was recorded for 1 min on a Hitachi double-beam spectrophotometer (model U-3200) and the enzyme activity was automatically calculated from the slope of the line as µmoles NADPH oxidized/min per ml.

All solutions and buffers were prepared using deionized, distilled, water. Solutions, except the phosphate buffer and EDTA, were freshly prepared each day. EDTA serves to inhibit the autoxidation of GSH in the reaction mixture (Cohen and Hochstein, 1963). Enzymes were diluted with 0.01 M potassium phosphate buffer (pH 7.0) so that the amount of enzyme added to the assay system provided an absorbance change of less than or equal to 0.05/min (0.83 µg Y_cY_{fetus} isoenzyme and 1.58 µg Y_cY_c isoenzyme). Varying concentrations of cumene hydroperoxide (80% in cumene) were prepared in 95% (v/v) ethanol. This resulted in a total ethanol concentration in the assay of less than 1%.

6.2.2. Glutathione S-Transferase Assay

GSH S-T activity was measured with CDNB as previously described (Chapter 2, Section 2.2.3.).

6.2.3. Kinetic and Inhibitor Studies

The initial rates of the GSH peroxidase reaction were measured at a cumene hydroperoxide concentration of 0.5 mM for varying concentrations of GSH, and at a GSH concentration of 1.0 mM for varying concentrations of cumene hydroperoxide. The kinetic data was analysed by use of a non-linear regression program, ENZFITTER (Elsevier-BIOSOFT, Cambridge, United Kingdom), written for the IBM PC computer.

In the GSH peroxidase inhibitor studies, 10 µl of an appropriate amount of the inhibitor was added to the reaction mixture just prior to the 10 min incubation period. These additions did not significantly affect the pH of the reaction mixture. Oxidized GSH (GSSG) (0.1 mM) was added to cuvettes following reactions in which inhibition was observed to ensure that GSH reductase had not been inhibited, and consequently proving the limiting factor in the assay.

The effects of ionic strength on inhibition were determined by increasing the KCl concentration in the potassium phosphate buffer used in the reaction mixture (see Results, Section 6.3.3., for details).

The GSH S-T inhibitor studies using CDNB as enzyme substrate were performed as previously described for acrolein (Chapter 5, Section 5.2.3.).

Acrolein and rose bengal (Sigma Chemical Company) solutions were prepared in ethanol (95%) and BSP (Sigma Chemical Company) solutions were prepared in H_2O. For the kinetic and inhibitor studies, the assays were performed in triplicate. The concentration of inhibitor resulting in 50% inhibition of enzyme activity, the I_{50} value, was determined from plots of remaining activity versus inhibitor concentration. Control values were obtained by pre-incubation with an appropriate volume of solvent in the absence of inhibitor.

6.3. RESULTS

6.3.1. Catalytic Properties

The velocity versus substrate concentration curves for both isoenzymes give the hyperbola typical for reactions obeying Michaelis-Menten kinetics (Dixon and Webb, 1979) (Figs. 6.1 and 6.2). Double reciprocal or Lineweaver-Burk (Lineweaver and Burk, 1934) plots for the reactions show expected linearity (Figs. 6.3 and 6.4). The apparent K_m and V_{max} values for GSH are given in Table 6.1;

Table 6.1. **APPARENT K_m and V_{max} VALUES FOR GSH**

Isoenzyme	K_m (mM)	V_{max} (µmol/min/mg)
Y_cY_{fetus}	0.134	7.27
Y_cY_c	0.105	4.20

and for cumene hydroperoxide are given in Table 6.2.

Table 6.2. **APPARENT K_m and V_{max} VALUES FOR CUMENE HYDROPEROXIDE**

Isoenzyme	K_m (mM)	V_{max} (µmol/min/mg)
Y_cY_{fetus}	0.169	9.77
Y_cY_c	0.842	8.06

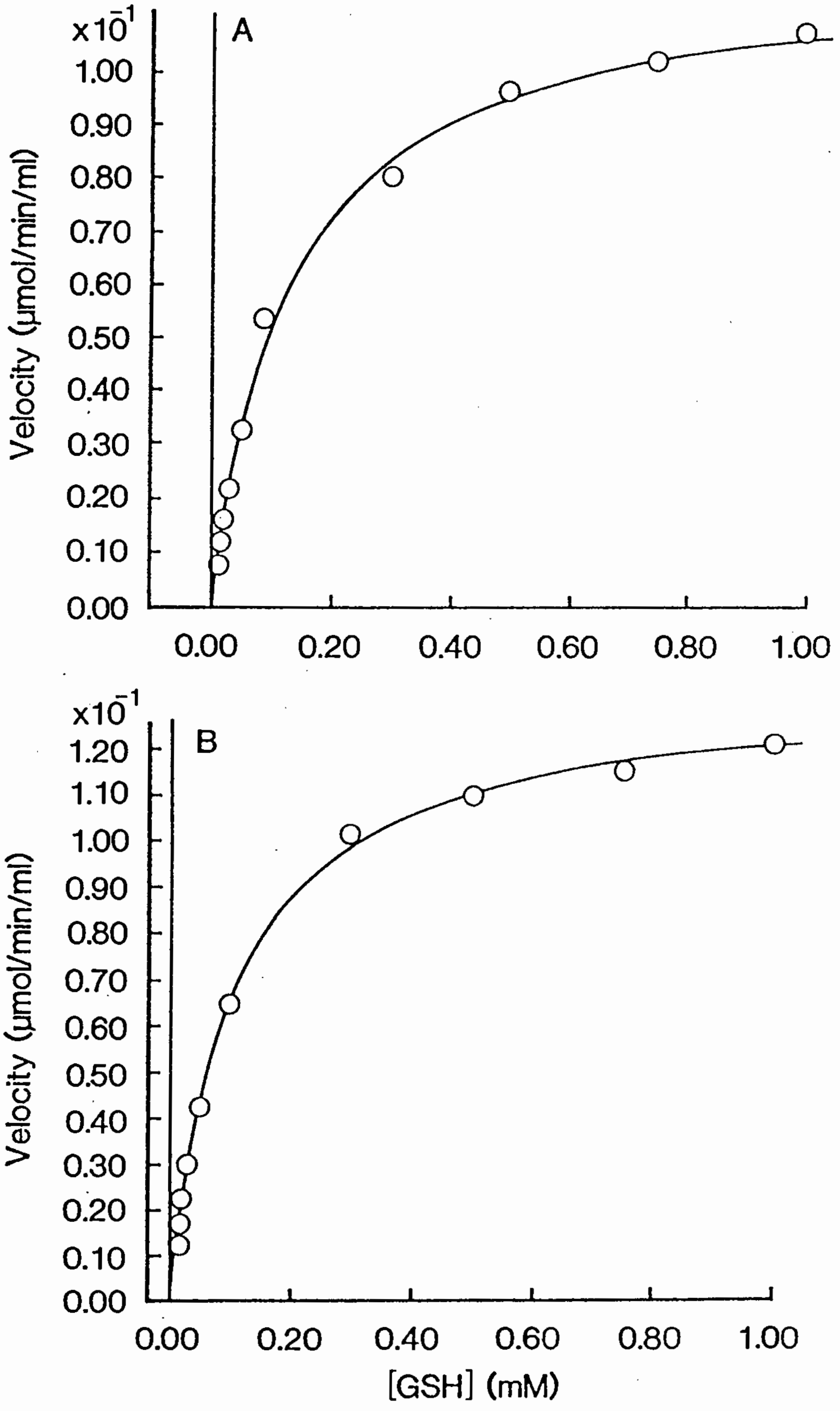


Fig. 6.1. **Velocity versus substrate concentration curves for the GSH peroxidase reaction as catalyzed by GSH S-T Y_cY_{fetus} (A) and GSH S-T Y_cY_c (B). Activity was measured at a constant cumene hydroperoxide concentration of 0.5 mM and concentrations of GSH were varied.**

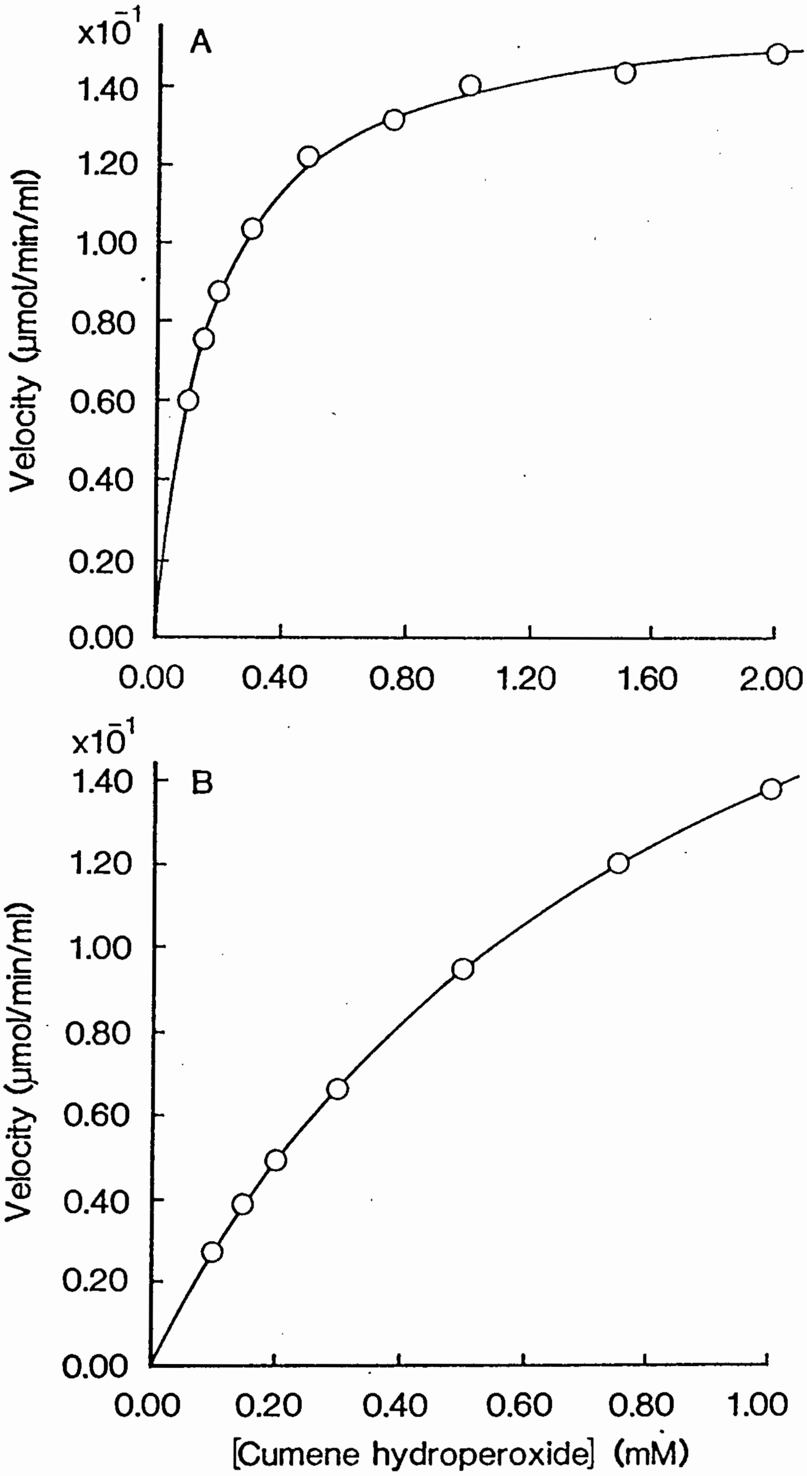


Fig. 6.2. **Velocity versus substrate concentration curves for the GSH peroxidase reaction as catalyzed by GSH S-T Y_cY_{fetus} (A) and GSH S-T Y_cY_c (B). Activity was measured at a constant GSH concentration of 1.0 mM and concentrations of cumene hydroperoxide were varied.**

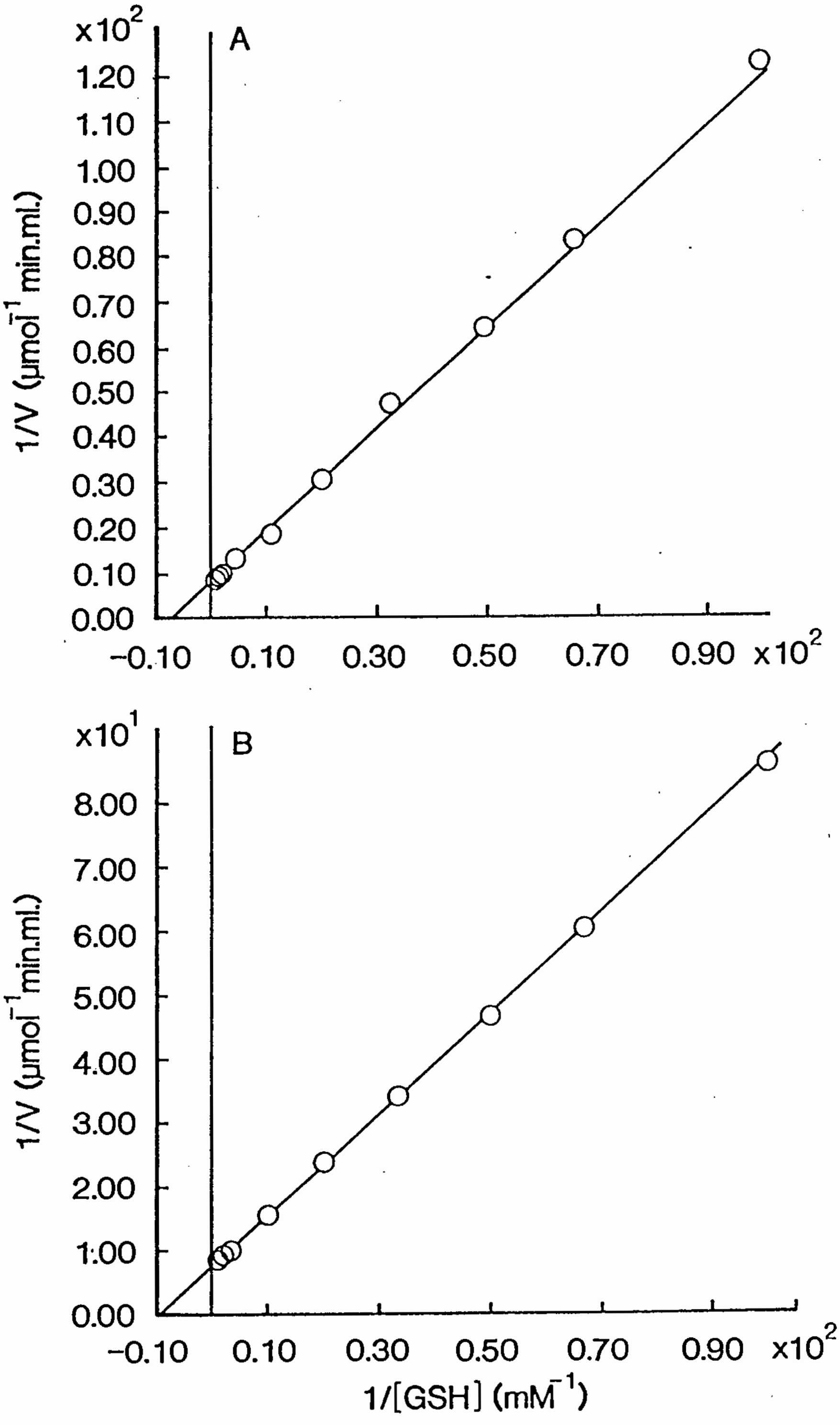


Fig. 6.3. **Lineweaver-Burk plots for the GSH peroxidase reaction as catalyzed by GSH S-Y Y_cY_{fetus} (A) and GSH S-T Y_cY_c (B). Activity was measured at a constant cumene hydroperoxide concentration of 0.5 mM and concentrations of GSH were varied.**

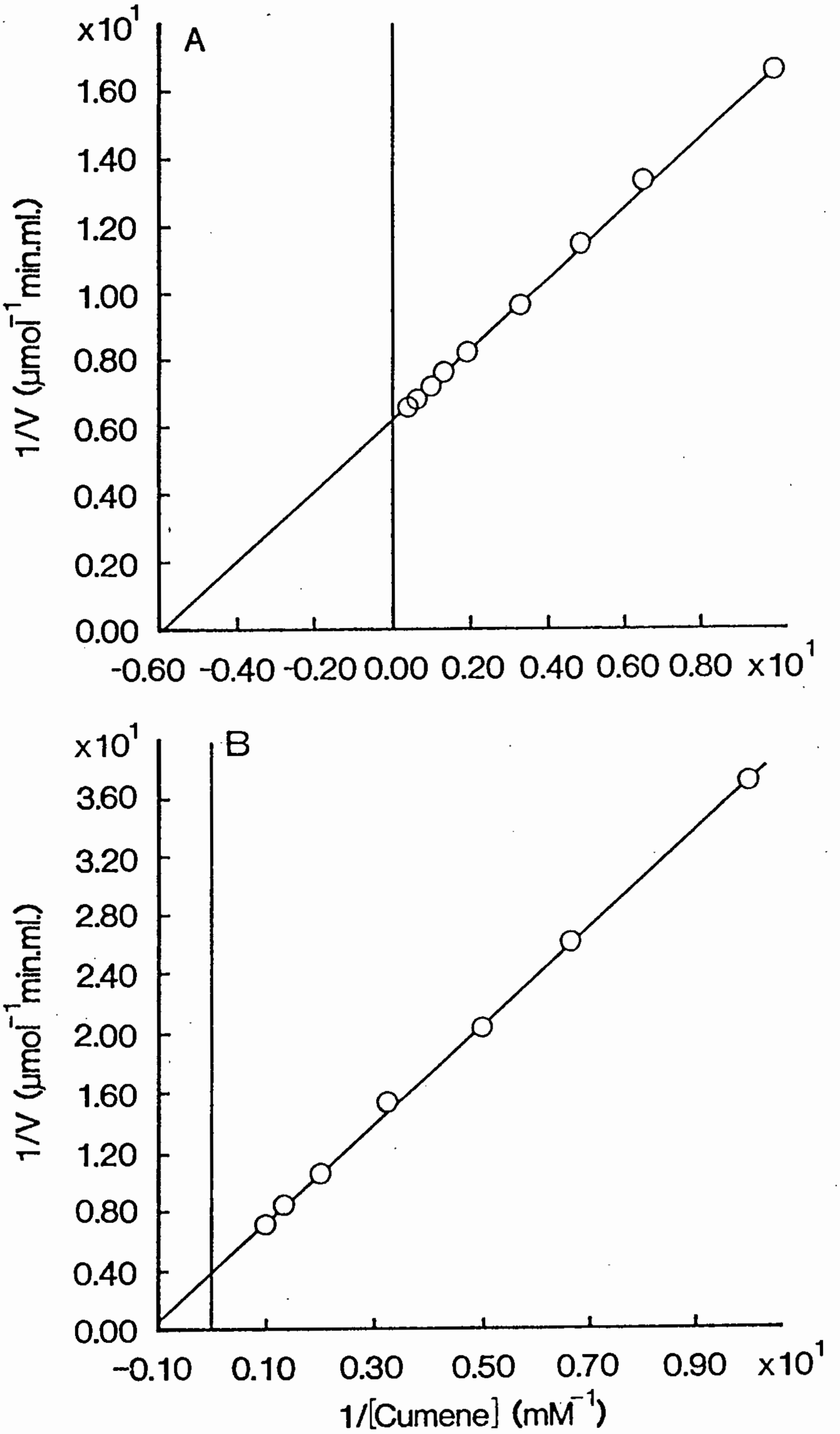


Fig. 6.4. **Lineweaver-Burk plots for the GSH peroxidase reaction as catalyzed by GSH S-Y Y_cY_{fetus} (A) and GSH S-T Y_cY_c (B). Activity was measured at a constant GSH concentration of 1.0 mM and concentrations of cumene hydroperoxide were varied.**

The catalytic efficiency (expressed as K_{cat}/K_m) (Alin et al, 1985a) of the two isoenzymes for cumene hydroperoxide was determined with 0.1 mM cumene hydroperoxide, at which concentration first-order kinetics apply. Under the assumption of Michaelis-Menten kinetics, the initial rate divided by the product of the total enzyme and substrate concentrations equals K_{cat}/K_m. This ratio was determined at a GSH concentration of 1 mM i.e. at saturating concentrations of GSH. The catalytic efficiency of the two isoenzymes for cumene hydroperoxide are given in Table 6.3.

Table 6.3. CATALYTIC EFFICIENCY FOR CUMENE HYDROPEROXIDE

Isoenzyme	K_{cat}/K_m ($mM^{-1}s^{-1}$)
Y_cY_{fetus}	64
Y_cY_c	16

The results show that the fetal isoenzyme has a catalytic efficiency for the peroxide substrate which is four fold higher than that of the adult isoenzyme, GSH S-T Y_cY_c.

6.3.2. Fulfillment of Coupled Assay Criteria

Under Section 6.2.1. the conditions necessary for a valid coupled assay were mentioned. To ensure that these conditions were met, inhibitor studies were performed at a GSH concentration of 1 mM i.e. $[GSH]_0 >> K_{mGSH}$, and a cumene hydroperoxide concentration of 1.2 mM. To confirm that GSH reductase was in excess, the amount of protein in the assay was decreased from 4 to 2 µg. This produced no change in the specific activity of the two isoenzymes for cumene hydroperoxide. Under the assay conditions, after a short lag period of approximately 0.3 min, the rate of disappearance of NADPH was linear for both isoenzymes over a period of approximately 5 min. Fig. 6.5 shows a profile printout for the reaction from the spectrophotometer over a 10 min period with GSH S-T Y_cY_{fetus} as GSH peroxidase source. After a period of approximately 5.5 min, the NADPH in the enzyme catalyzed reaction has been utilized and the absorbance begins to increase when measured against the blank.

6.3.3. The Effect of Ionic Strength, pH, Incubation Time and Temperature on the Glutathione Peroxidase Assay

Fig. 6.6 shows the effect of incubation time and temperature on the GSH peroxidase activity of GSH S-T Y_cY_{fetus}. GSH peroxidase activity increases with incubation time to a maximum at a time of 10 mins and then decreases over the next 10 mins. At 37°C the non-enzymatic rate is higher than at 25°C and hence the lower GSH peroxidase activity obtained. Similar results were obtained for the Y_cY_c isoenzyme.

The effect of pH on the enzymatic reaction showed that the non-enzymatic reaction was strongly pH dependent. At pH 6 the GSH peroxidase activity was low and increased dramatically at a pH of 7. At

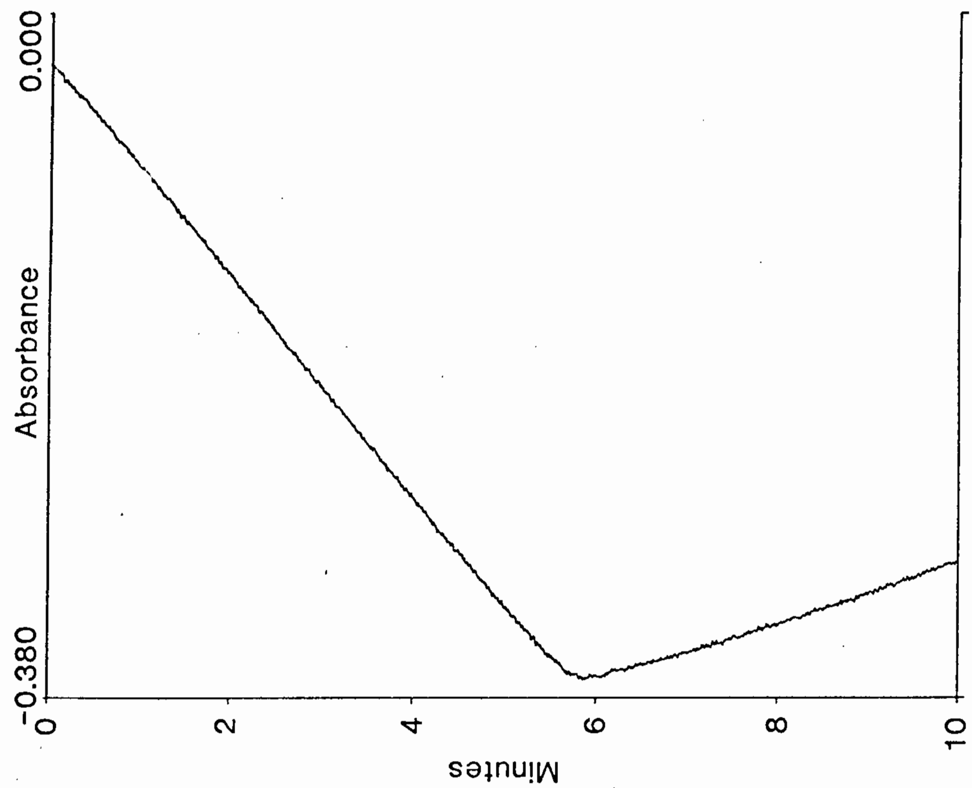


Fig. 6.5. Profile printout from the spectrophotometer for the GSH peroxidase reaction as catalyzed by GSH S-T Y_cY_{fetus}. Activity was measured at GSH and cumene hydroperoxide concentrations of 1.0 and 1.2 mM respectively.

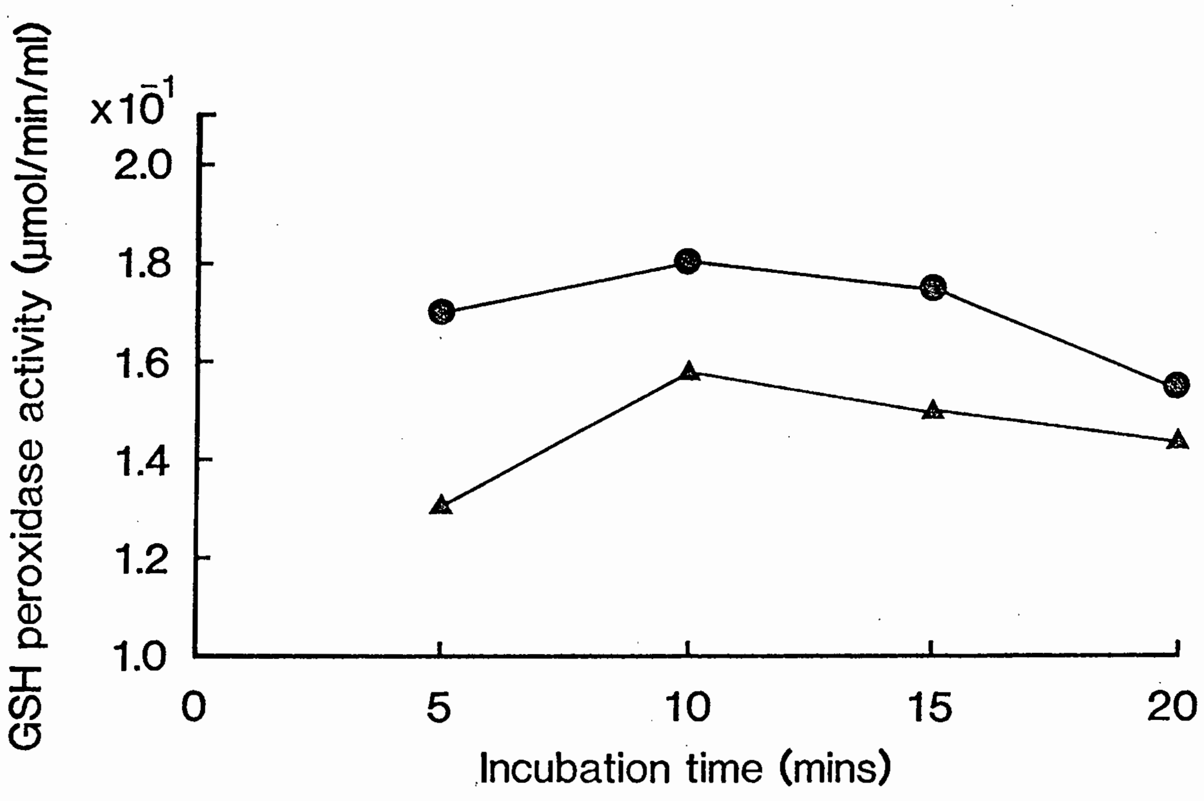


Fig. 6.6. Graph of activity versus incubation time for the GSH peroxidase assay as catalyzed by GSH S-T Y_cY_{fetus}. Activities were determined at 25°C (●) and 37°C (▲).

pH 8 the non-enzymatic rate was found to be very high and consequently a pH value of 7 was considered to provide an even balance between the spontaneous and enzyme catalyzed reaction rates.

Fig. 6.7 shows the effect of ionic strength on the GSH peroxidase activity of GSH S-T Y_cY_{fetus}. Similar results were obtained for the Y_cY_c isoenzyme. The KCl concentrations in the potassium phosphate buffer were 0, 0.1, 0.2, 0.3 and 0.5 M which corresponded to a total KCl concentration in the reaction mixture of 0, 54, 108, 162 and 270 mM respectively. Maximum activities were obtained at KCl concentrations of 54 to 108 mM and then decreased as the KCl concentration was further increased. Because there were no marked differences between the GSH peroxidase activity obtained at 54 or 108 mM KCl, for standardization purposes, a KCl concentration of 54 mM (used for catalytic studies) was used as the standard in all GSH peroxidase assays.

As GSH reductase is present in excess, the effects discussed above are more likely to be a function of the GSH peroxidase present in the assay, than the GSH reductase.

6.3.4. The Effect of BSP on Enzyme Activity

6.3.4.1. Effect on Glutathione Peroxidase Activity

Fig. 6.8 shows the effects of increasing BSP concentrations on the GSH peroxidase activity of GSH S-T Y_cY_{fetus} and Y_cY_c. The I_{50} values for the two isoenzymes are given in Table 6.4.

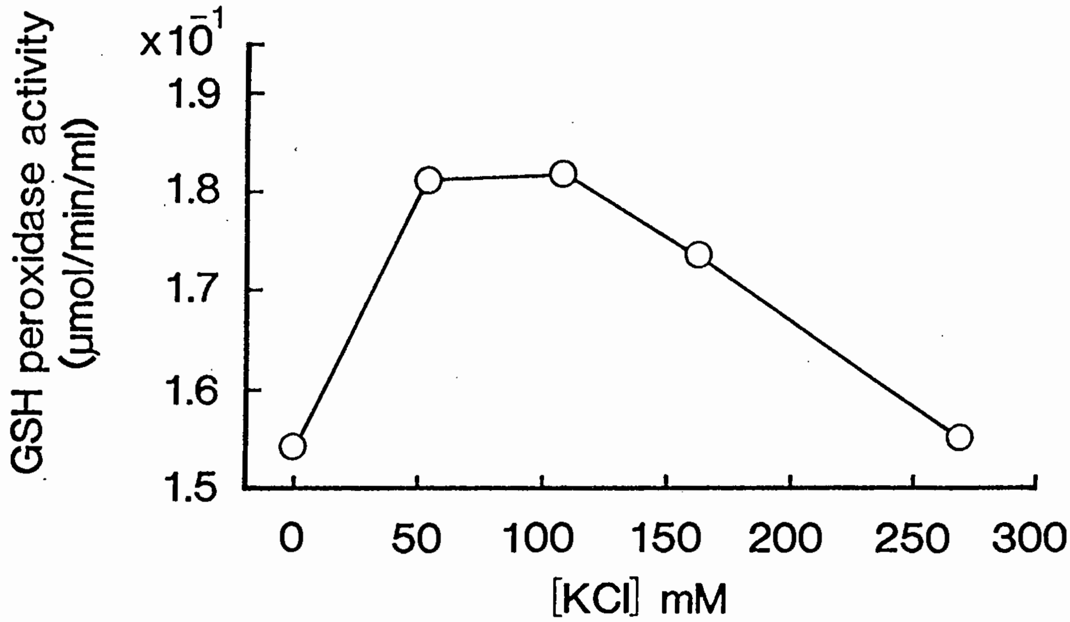


Fig. 6.7. Graph of activity versus KCl concentration for the GSH peroxidase assay as catalyzed by GSH S-T Y_cY_{fetus}. The KCl concentrations in the potassium phosphate incubation buffer were 0, 0.1, 0.2, 0.3 and 0.5 M which corresponded to a total added KCl concentration in the assay of 0, 54, 108, 162 and 270 mM respectively.

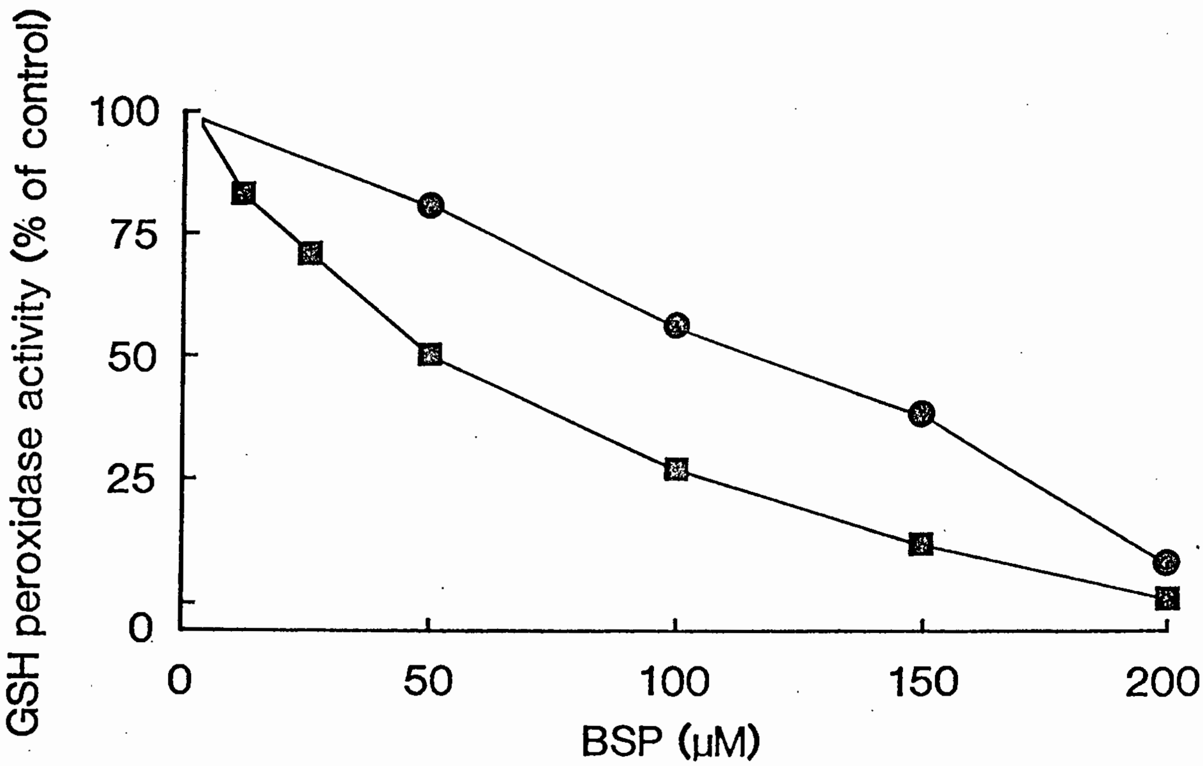


Fig. 6.8. Inhibition of the GSH peroxidase activity of GSH S-T Y_cY_{fetus} (■) and Y_cY_c (●) by varying concentrations of BSP.

Table 6.4. **I_{50} VALUE FOR BSP INHIBITION OF GSH PEROXIDASE ACTIVITY**

Isoenzyme	I_{50} (µM)
Y_cY_{fetus}	50
Y_cY_c	116

Figs. 6.9 and 6.10 show the effects of ionic strength on BSP inhibition of the two isoenzymes. In the presence of 270 mM KCl the I_{50} for the fetal isoenzyme increases from 50 to 70 µM (Fig. 6.9). However, as the concentration of BSP increases these effects are reversed and at BSP concentrations above 100 µM the fetal isoenzyme becomes more susceptible to inhibition at the higher ionic strength.

GSH S-T Y_cY_c is more susceptible to inhibition in the presence of higher KCl concentrations (Fig. 6.10). In the presence of 270 mM KCl the I_{50} decreases from 116 to 76 µM. This increased susceptibility to inhibition is exhibited over the entire BSP concentration range. Table 6.5 (data taken from Figs. 6.9 and 6.10) shows the effects of KCl concentration on the inhibition of GSH S-T Y_cY_{fetus} and Y_cY_c by 50 and 100 µM BSP respectively.

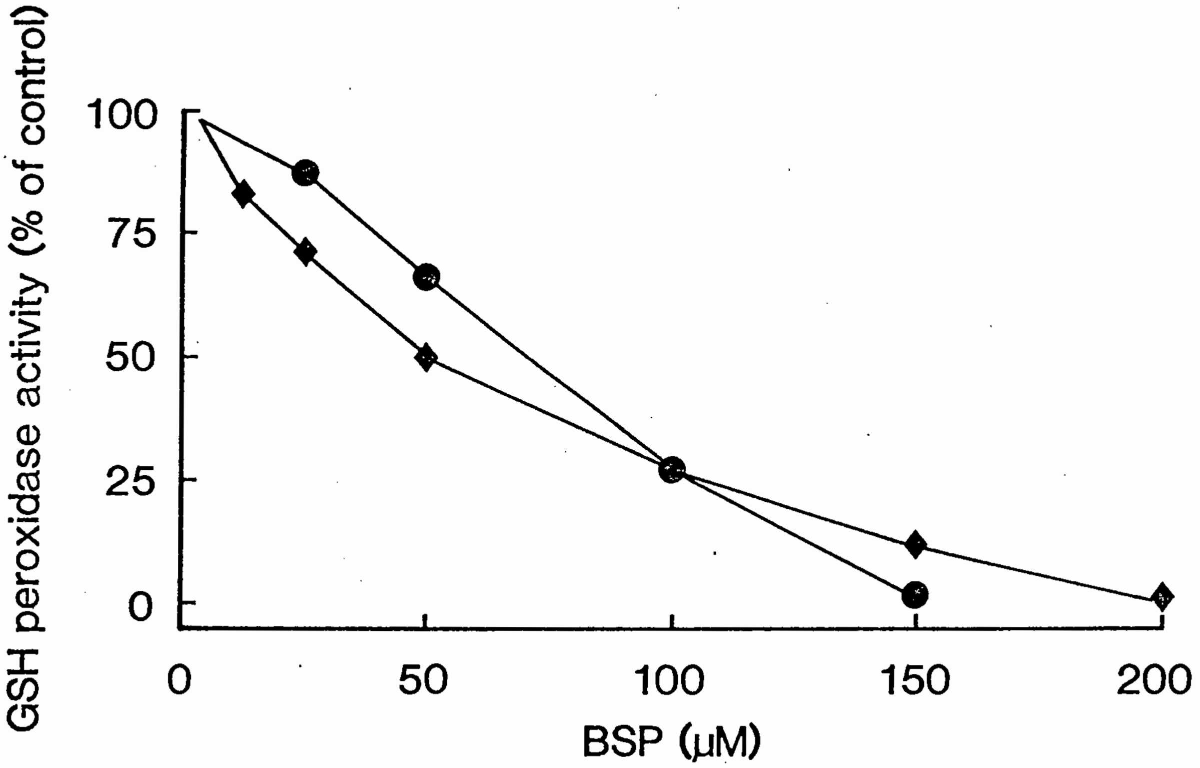


Fig. 6.9. Inhibition of the GSH peroxidase activity of GSH S-T Y_cY_{fetus} by varying concentrations of BSP in the presence of 54 (◆) and 270 mM (●) KCl.

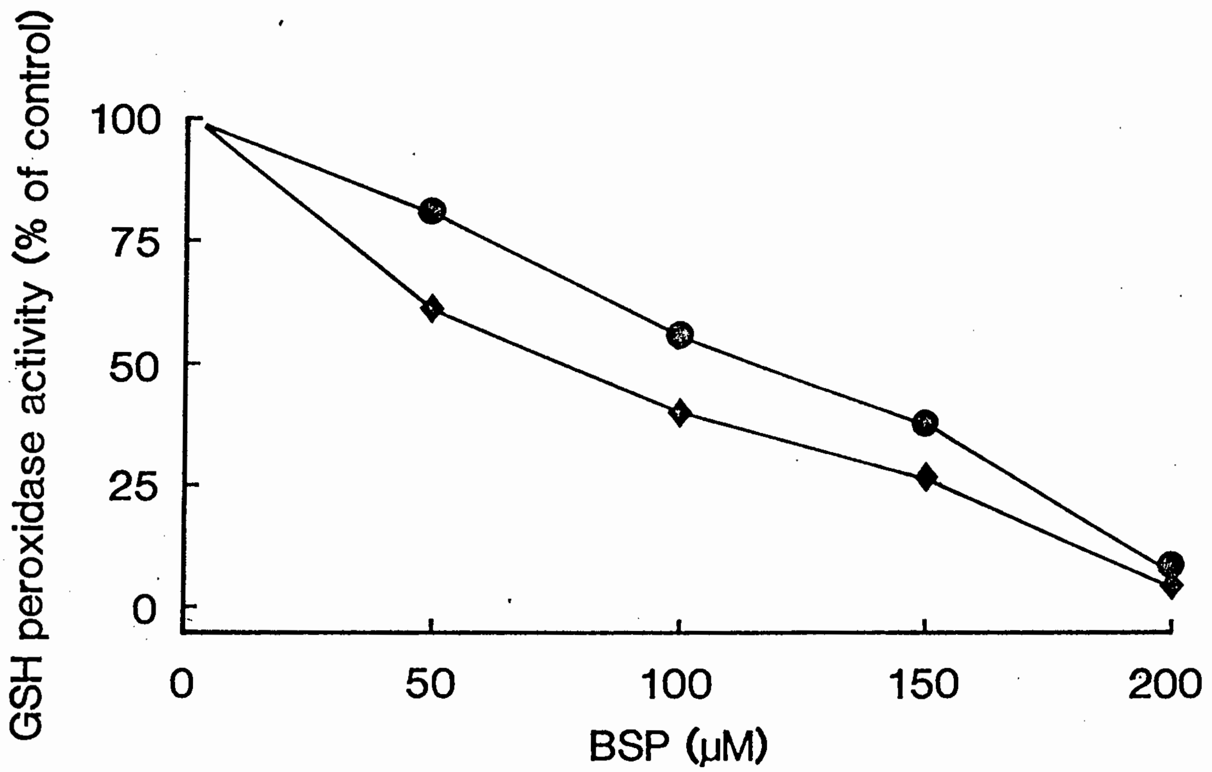


Fig. 6.10. **Inhibition of the GSH peroxidase activity of GSH S-T Y_cY_c by varying concentrations of BSP in the presence of 54 (●) and 270 mM (◆) KCl.**

Table 6.5. **EFFECT OF KCl CONCENTRATION ON BSP INHIBITION OF GSH PEROXIDASE ACTIVITY**

Isoenzyme	BSP Concn. (μM)	KCl Concn. (mM)	Inhibition (%)
Y_cY_{fetus}	50	54	50
	50	270	34
Y_cY_c	100	54	44
	100	270	60

Fig. 6.11 shows a profile printout from the spectrophotometer illustrating that BSP inhibition in the assay is not a consequence of GSH reductase inhibition. "A" shows the normal reaction profile for the fetal isoenzyme. "B" shows the profile in the presence of 200 μM BSP. "C" shows the profile obtained after the addition of 0.1 mM oxidized GSH (GSSG) to the inhibited assay. This illustrates that GSSG is the limiting factor in the assay i.e. GSH reductase has not been inhibited. This was similarly confirmed for the Y_cY_c isoenzyme.

6.3.4.2. Effect on GSH S-Transferase Activity

Fig. 6.12 shows the effect of BSP on the GSH S-T activity of the two isoenzymes. The I_{50} values for GSH S-T Y_cY_{fetus} and Y_cY_c are given in Table 6.6.

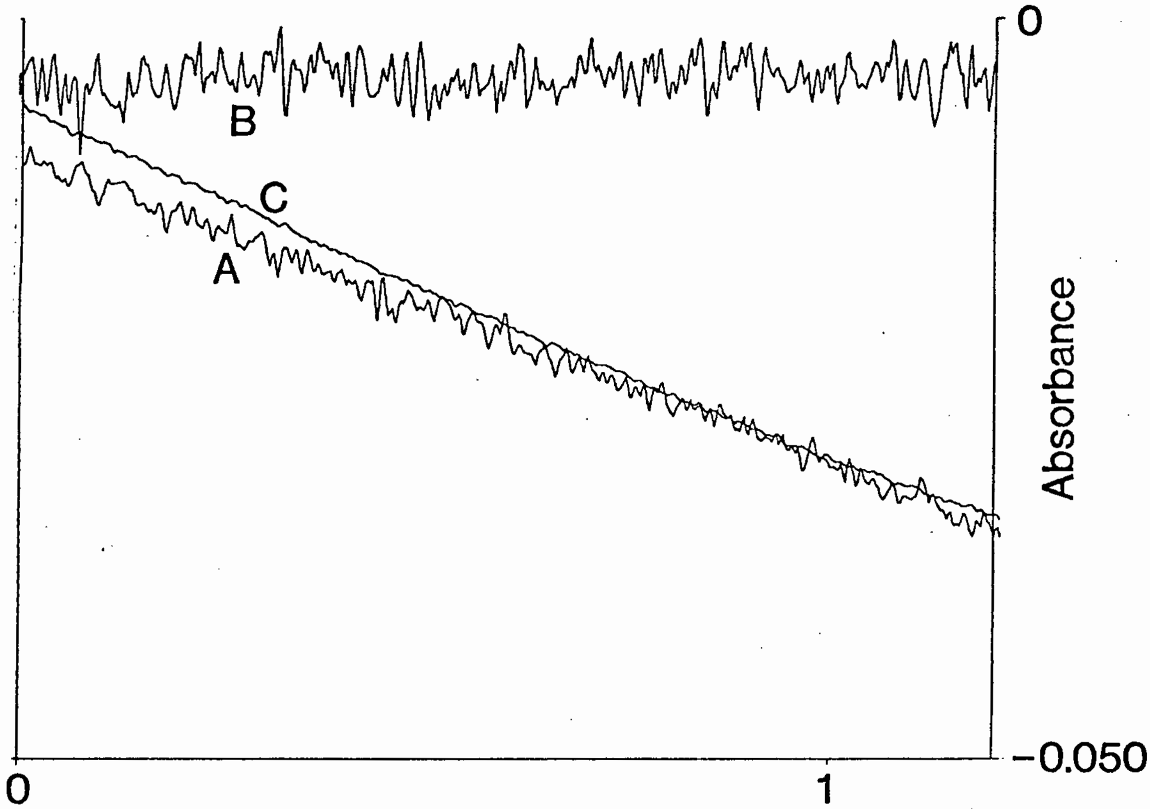


Fig. 6.11. Profile printout from the spectrophotometer illustrating the inhibition of GSH S-T Y_cY_{fetus} by 200 µM BSP ("B"). "A" shows the normal reaction profile in the absence of inhibitor and "C" shows the profile obtained after the addition of 0.1 mM oxidized GSH (GSSG) to the inhibited assay.

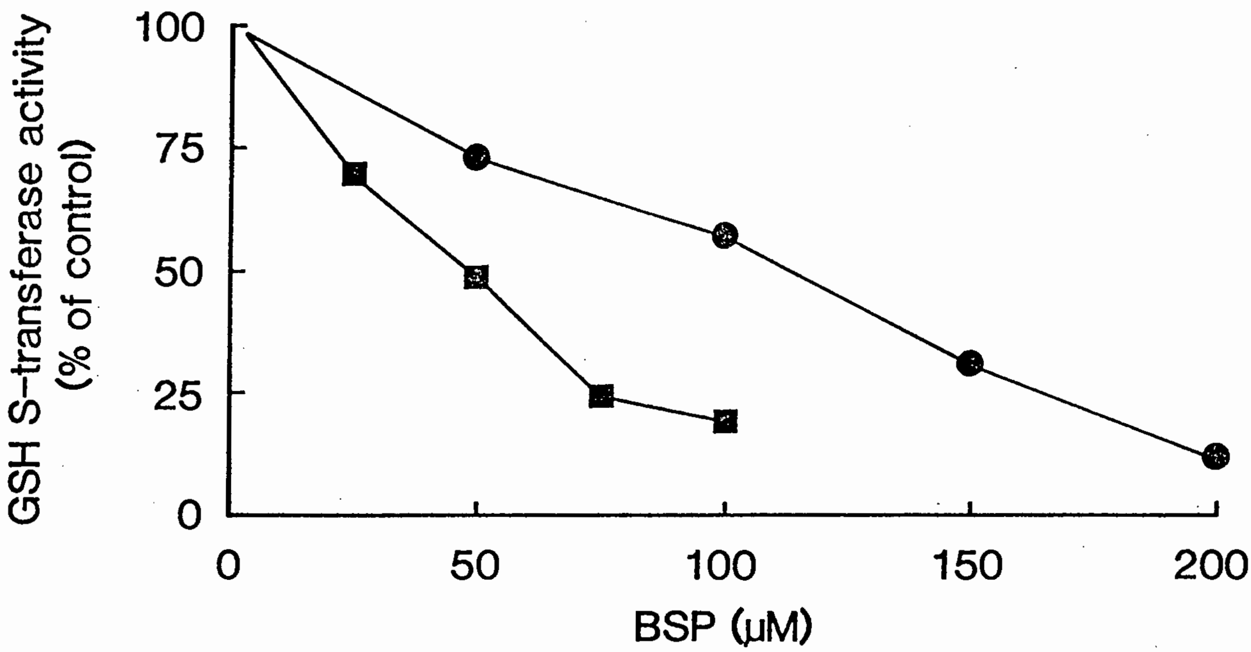


Fig. 6.12. Inhibition of the GSH S-T activity of transferases Y_cY_{fetus} (■) and Y_cY_c (●) by varying concentrations of BSP.

Table 6.6. **I_{50} VALUE FOR BSP INHIBITION OF GSH S-T ACTIVITY**

Isoenzyme	I_{50} (μM)
Y_cY_{fetus}	48
Y_cY_c	114

These I_{50} values are almost identical to those obtained for BSP inhibition of GSH peroxidase activity (Table 6.4).

Fig. 6.13 shows the effect of increasing ionic strength on BSP inhibition of GSH S-T Y_cY_{fetus}. The KCl concentration in the phosphate buffer was 0.5 M which corresponded to a total KCl concentration in the reaction mixture of 450 mM. The inhibition profile obtained is not dissimilar to that obtained for GSH peroxidase inhibition (see Fig. 6.9). At a KCl concentration of 450 mM the fetal isoenzyme was less susceptible to inhibition at the lower BSP concentrations. GSH S-T Y_cY_c was more susceptible to BSP inhibition in the presence of KCl. The percentage inhibition of GSH S-T Y_cY_{fetus} and Y_cY_c by 50 and 100 μM BSP respectively, is given in Table 6.7.

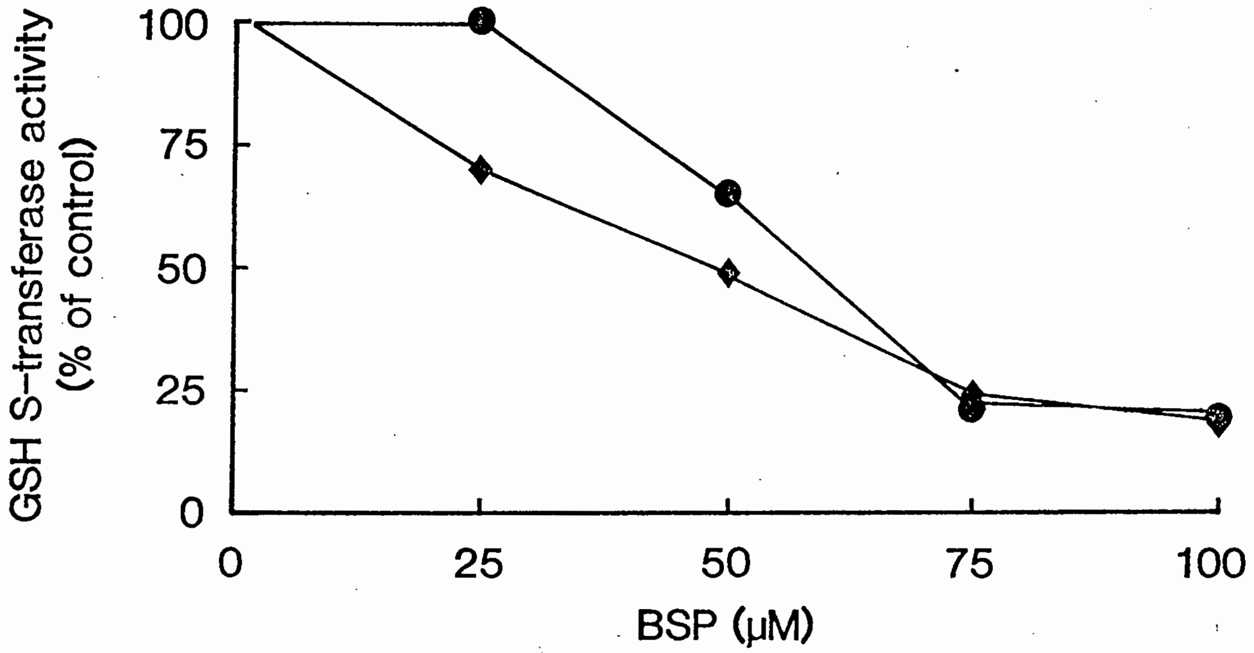


Fig. 6.13. Inhibition of the GSH S-T activity of transferase Y_cY_{fetus} by varying concentrations of BSP in the presence of 0 (◆) and 450 mM (●) KCl.

Table 6.7. **EFFECT OF KCl CONCENTRATION ON BSP INHIBITION OF GSH S-T ACTIVITY**

Isoenzyme	BSP Concn. (μM)	KCl Concn. (mM)	Inhibition (%)
Y_cY_{fetus}	50	0	51
	50	450	35
Y_cY_c	100	0	43
	100	450	64

These values are almost identical to those obtained for the effects of ionic strength on BSP inhibition of GSH peroxidase activity (Table 6.5).

Fig. 6.14 shows BSP inhibition of GSH peroxidase and GSH S-T activity for the two isoenzymes. The inhibition of enzymatic activity towards both substrates show very similar profiles for both the isoenzymes.

6.3.5. The Effect of Rose Bengal on Enzyme Activity

6.3.5.1. Effect on Glutathione Peroxidase Activity

Rose bengal was found to have no inhibitory effect on GSH reductase. Fig. 6.15 shows the effects of increasing concentrations of rose bengal on the GSH peroxidase activity of GSH S-T Y_cY_{fetus} and Y_cY_c. The I_{50} values for the two isoenzymes are given in Table 6.8.

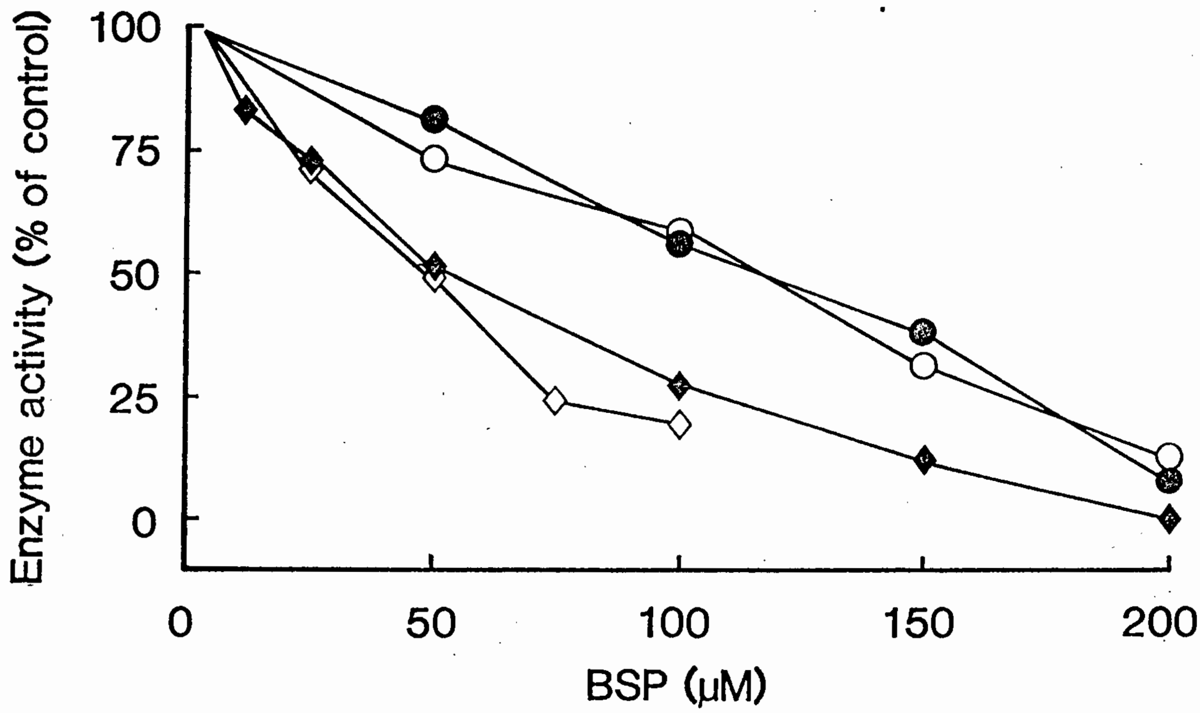


Fig. 6.14. Inhibition of enzyme activity by varying concentrations of BSP. Graph shows inhibition of the GSH peroxidase (◆) and GSH S-T (◇) activity of transferase Y_cY_{fetus}, and inhibition of the GSH peroxidase (●) and GSH S-T (○) activity of transferase Y_cY_c.

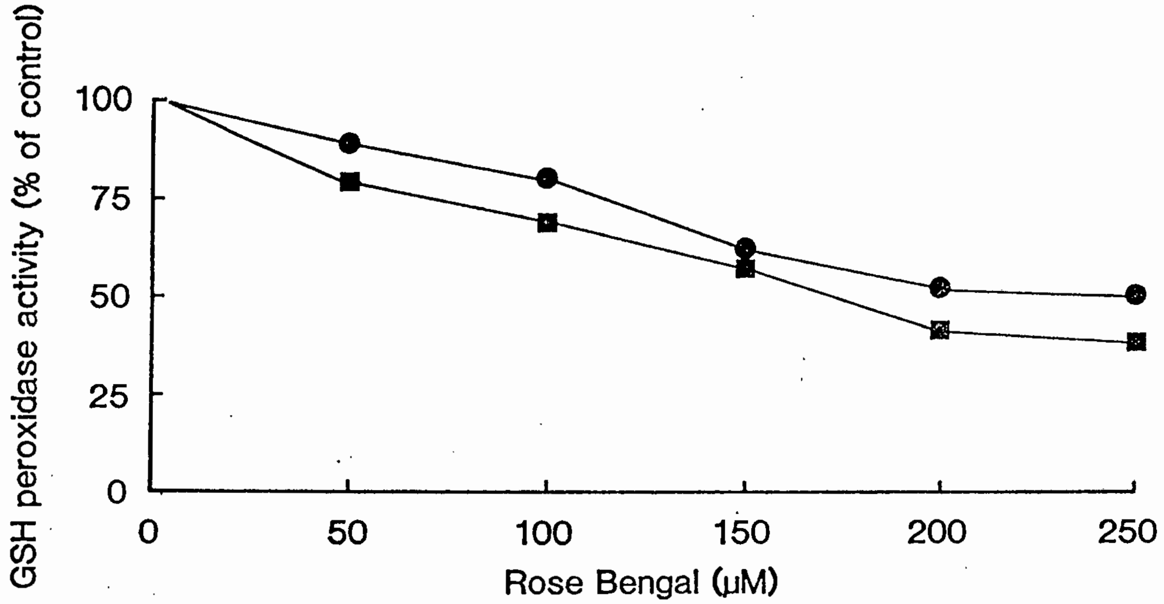


Fig. 6.15. Inhibition of the GSH peroxidase activity of GSH S-T Y_cY_{fetus} (■) and Y_cY_c (●) by varying concentrations of rose bengal.

Table 6.8. I_{50} VALUE FOR ROSE BENGAL INHIBITION OF GSH PEROXIDASE ACTIVITY

Isoenzyme	I_{50} (μM)
Y_cY_{fetus}	172
Y_cY_c	250

At a concentration above 200 μM there was no marked increase in the percentage inhibition with increasing concentration of non-substrate ligand.

Increasing the KCl concentration in the assay produced small changes in GSH peroxidase inhibition. The percentage inhibition of the two isoenzymes at a rose bengal concentration of 150 μM is given in Table 6.9.

Table 6.9. EFFECT OF KCl CONCENTRATION ON ROSE BENGAL INHIBITION OF GSH PEROXIDASE ACTIVITY

Isoenzyme	Rose Bengal Concn. (μM)	KCl Concn. (mM)	Inhibition (%)
Y_cY_{fetus}	150	54	43
	150	270	40
Y_cY_c	150	54	38
	150	270	37

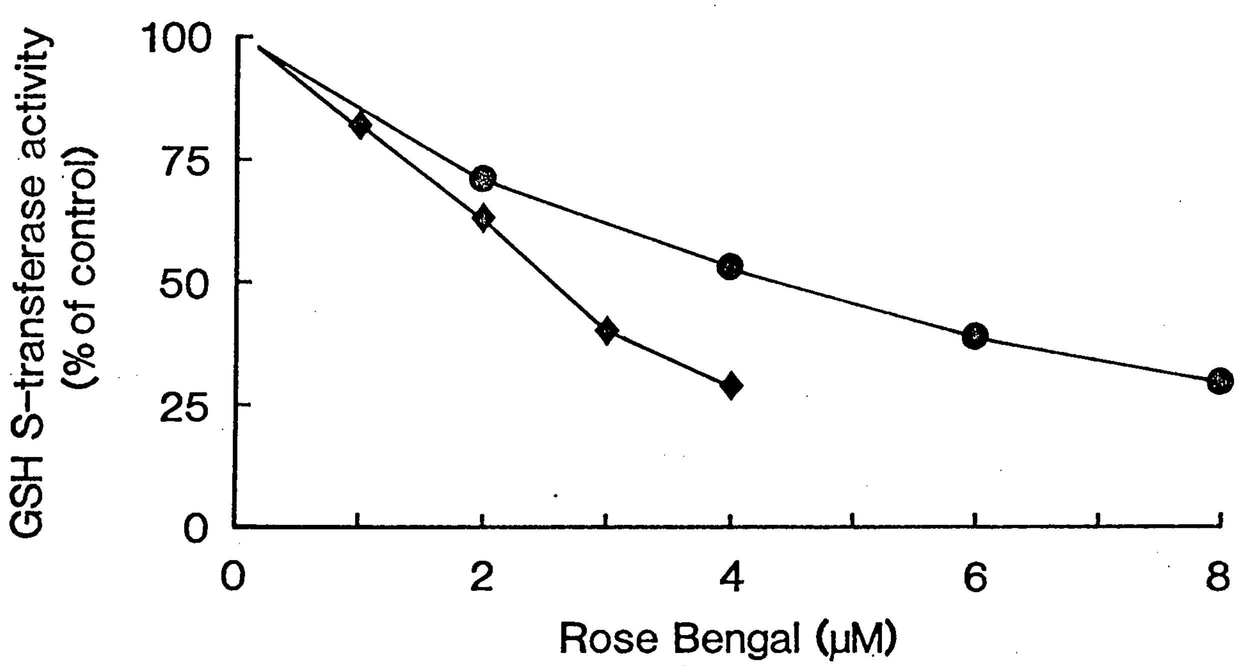


Fig. 6.16. **Inhibition of the GSH S-T activity of transferases Y_cY_{fetus} (◆) and Y_cY_c (●) by varying concentrations of rose bengal.**

6.3.6. The Effect of Acrolein on Enzyme Activity

6.3.6.1. Effect on Glutathione Peroxidase Activity

Acrolein was found to have no inhibitory effect on GSH reductase. Fig. 6.17 shows the effects of increasing concentrations of acrolein on the GSH peroxidase activity of GSH S-T Y_cY_{fetus} and Y_cY_c. The I_{50} values for the two isoenzymes are given in Table 6.12.

Table 6.12. I_{50} VALUE FOR ACROLEIN INHIBITION OF GSH PEROXIDASE ACTIVITY

Isoenzyme	I_{50} (mM)
Y_cY_{fetus}	0.65
Y_cY_c	0.33

Fig. 6.18 shows the effects of increasing KCl concentrations on the GSH peroxidase activity of the Y_cY_{fetus} isoenzyme. Increasing the KCl concentration in the assay resulted in an increase in GSH peroxidase inhibition for both isoenzymes. At an acrolein concentration of 0.4 mM, the percentage inhibition of the two isoenzymes with increasing KCl concentrations is given in Table 6.13.

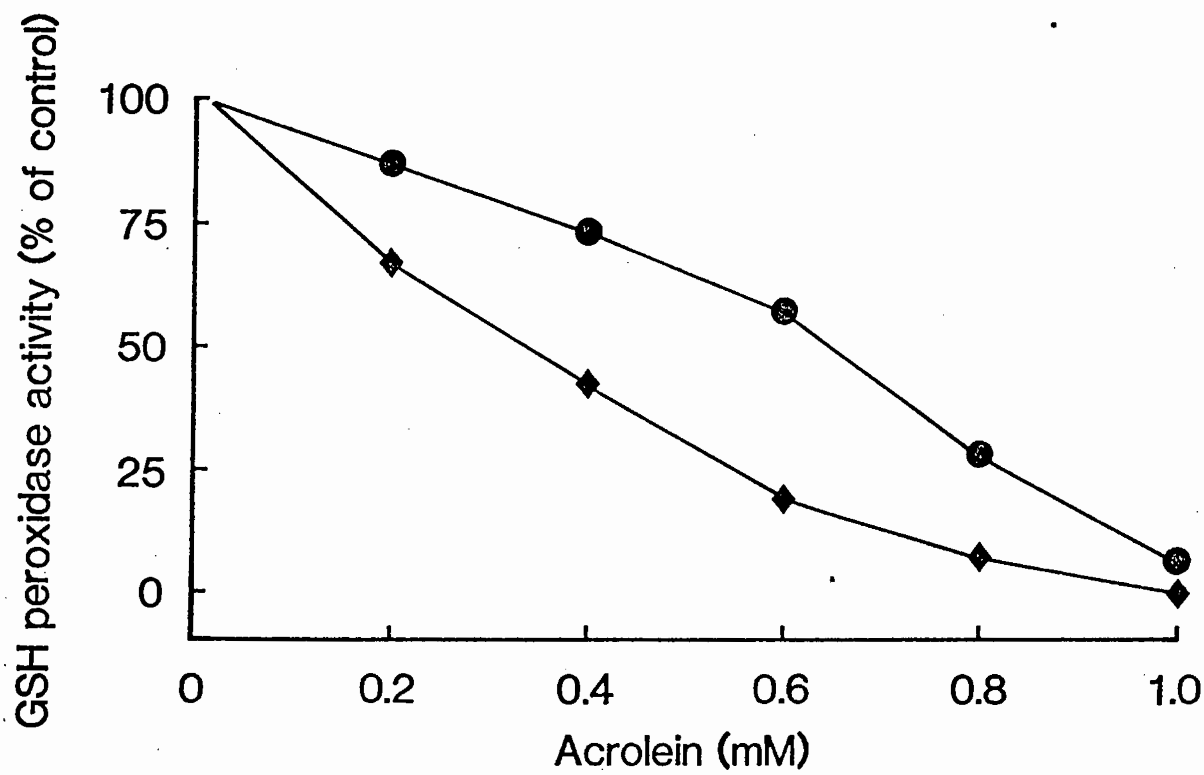


Fig. 6.17. **Inhibition of the GSH peroxidase activity of GSH S-T Y_cY_{fetus} (●) and Y_cY_c (◆) by varying concentrations of acrolein.**

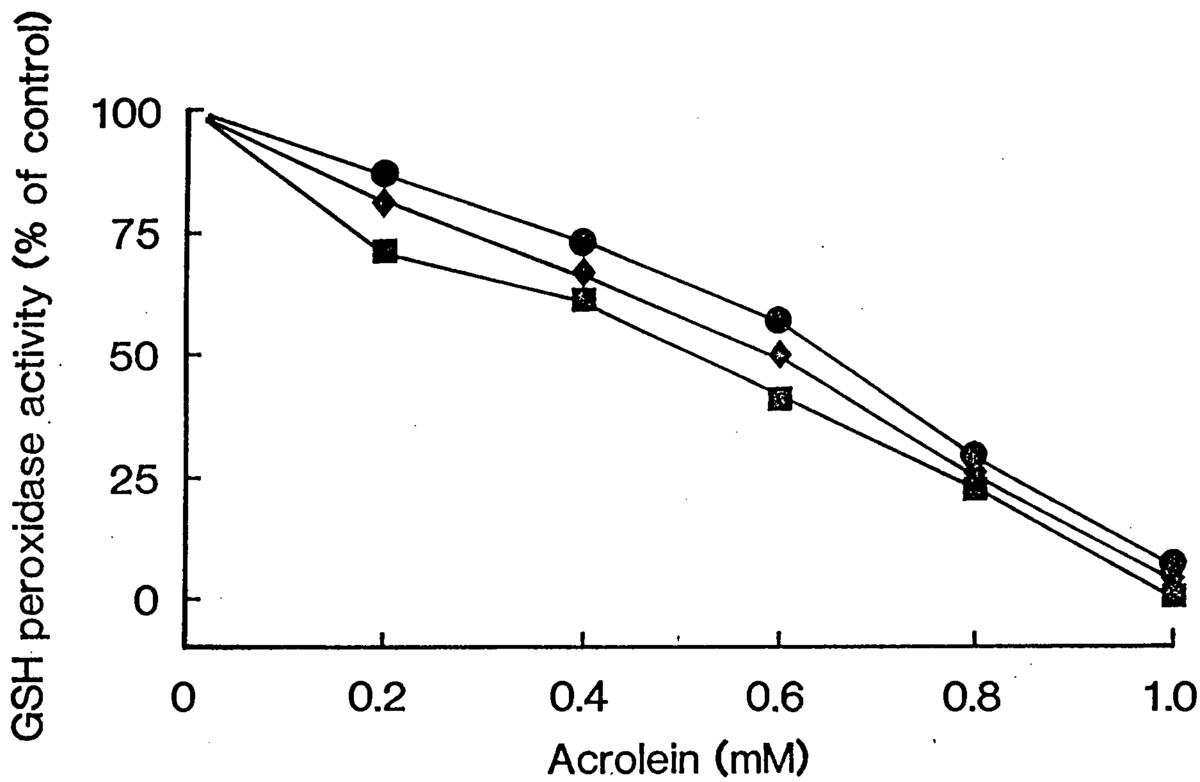


Fig. 6.18. **Inhibition of the GSH peroxidase activity of GSH S-T Y_cY_{fetus} by varying concentrations of acrolein in the presence of 54 (●), 108 (◆) and 270 mM (■) KCl.**

Table 6.13. **EFFECT OF KCl CONCENTRATION ON ACROLEIN INHIBITION OF GSH PEROXIDASE ACTIVITY**

Isoenzyme	Acrolein Concn. (mM)	KCl Concn. (mM)	Inhibition (%)
Y_cY_{fetus}	0.4	54	27
	0.4	108	33
	0.4	270	39
Y_cY_c	0.4	54	58
	0.4	108	60
	0.4	270	69

6.3.6.2. Effect on GSH S-Transferase Activity

Fig. 6.19 shows acrolein inhibition of the GSH peroxidase and GSH S-T activity of the two isoenzymes. The I_{50} values for the inhibition of GSH S-T activity of the two isoenzymes are given in Table 6.14.

Table 6.14. **I_{50} VALUE FOR ACROLEIN INHIBITION OF GSH S-T ACTIVITY**

Isoenzyme	I_{50} (mM)
Y_cY_{fetus}	0.7
Y_cY_c	0.8

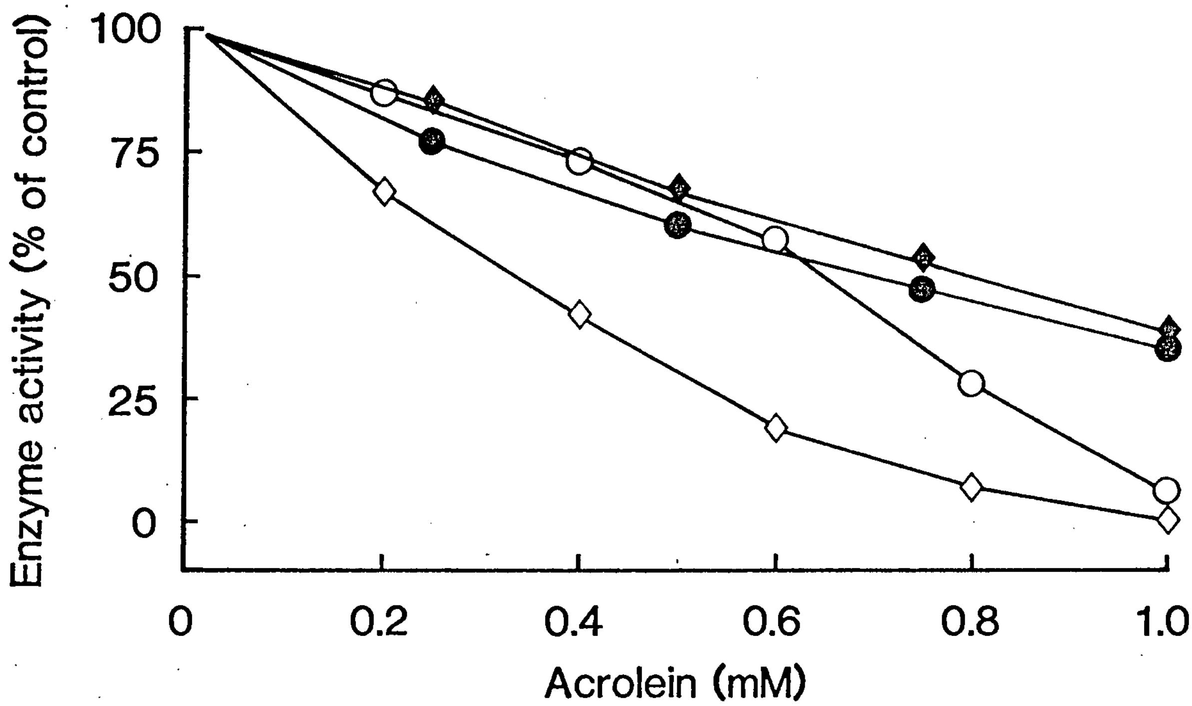


Fig. 6.19. Inhibition of enzyme activity by varying concentrations of acrolein. Graph shows inhibition of the GSH peroxidase (○) and GSH S-T (●) activity of transferase Y_cY_{fetus}, and inhibition of the GSH peroxidase (◇) and GSH S-T (◆) activity of transferase Y_cY_c.

Increasing the KCl concentration in the assay produced small changes in GSH S-T inhibition. At acrolein concentrations of 0.3 and 0.8 mM, the percentage inhibition of the two isoenzymes is given in Table 6.15.

Table 6.15. **EFFECT OF KCl CONCENTRATION ON ACROLEIN INHIBITION OF GSH S-T ACTIVITY**

Isoenzyme	Acrolein Concn. (mM)	KCl Concn. (mM)	Inhibition (%)
Y_cY_{fetus}	0.3	0	25
	0.3	450	26
	0.8	0	45
	0.8	450	47
Y_cY_c	0.3	0	18
	0.3	450	21
	0.8	0	52
	0.8	450	54

6.4. DISCUSSION

Analysis of the catalytic properties of the two isoenzymes with cumene hydroperoxide as substrate (Section 6.3.1.) show that both isoenzymes obey Michaelis-Menten kinetics. Prohaska (1980) has previosly reported similar results for GSH S-T Y_cY_c. The apparent K_m values of both isoenzymes for GSH do not show a marked difference. However, the K_m value of the Y_cY_c isoenzyme for cumene hydroperoxide is approximately five fold higher than that of the fetal isoenzyme. A low K_m is regarded as an advantage for a substrate present in low concentrations, as this enables the enzyme concerned to catalyze the reaction rapidly (Lehninger, 1977). An analysis of the catalytic efficiency (K_{cat}/K_m) of the two isoenzymes confirms that the fetal isoenzyme is approximately four times more efficient in catalyzing the reduction of cumene hydroperoxide (Table 6.3).

BSP is a substrate for the GSH S-T and can inhibit the activity of the GSH S-T with other substrates (Pabst et al, 1974; Vander Jagt et al, 1982; Alin et al, 1985b). The results of the effect of BSP on enzyme activity (6.3.4.) show that the ligand inhibits both the GSH peroxidase and the GSH S-T activity of the two isoenzymes. Lawrence et al (1978) found that BSP inhibited the GSH peroxidase activity of GSH S-T B and the present results confirm that this occurs with other GSH S-T. The I_{50} values for inhibition of both GSH peroxidase and GSH S-T activity are almost identical for the respective isoenzymes (Table 6.16 and Fig. 6.14). This suggests that BSP is able to block the catalytic site(s) of both isoenzymes towards both substrates in a similar manner. The fetal

Table 6.16. **I_{50} VALUES FOR THE INHIBITION OF THE GSH PEROXIDASE AND GSH S-T ACTIVITIES OF GSH S-T Y_cY_{fetus} AND Y_cY_c FOLLOWING INHIBITION BY BSP, ROSE BENGAL AND ACROLEIN**

Inhibitor	Y_cY_{fetus} I_{50} (μM)		Y_cY_c I_{50} (μM)	
	GSH Perox.	GSH S-T	GSH Perox.	GSH S-T
BSP	50	48	116	114
Rose Bengal	172	2.6	250	4.4
Acrolein	650	700	330	800

Enzyme inhibition was measured with cumene hydroperoxide and CDNB as enzyme substrates as described under Methods (6.2.3.). Table is a duplication of data from Results (6.3.4., 6.3.5. and 6.3.6.).

isoenzyme exhibited a higher susceptibility to BSP inhibition than the Y_cY_c isoenzyme (Table 6.16). Alterations of the ionic conditions in the assay produced changes in the inhibition profiles of both isoenzymes towards both substrates. These profiles for inhibition of both the GSH peroxidase and GSH S-T activity of the fetal isoenzyme are not dissimilar (Figs. 6.9 and 6.13). Similar results were obtained for the Y_cY_c isoenzyme (Table 6.5 and Table 6.7). This suggests that ionic factors play a role in the binding of BSP to the two isoenzymes.

The inhibition of enzyme activity by rose bengal (Section 6.3.5.) differs markedly from BSP inhibition. A major difference being observed between the I_{50} values obtained for GSH peroxidase inhibition and those obtained for GSH S-T inhibition (Table 6.16). The GSH peroxidase activity of both isoenzymes is approximately sixty fold more resistant to inhibition than the GSH S-T activity. The increased susceptibility of the GSH S-T activity of both isoenzymes to rose bengal inhibition, could be due to either a pH or ionic effect. The latter seems unlikely as a high ionic strength (450 mM KCl) failed to produce major changes in the percentage inhibition (Table 6.11). Alterations in pH have been shown to influence the binding of non-substrate ligands to the GSH S-T (Tipping et al, 1976) and influence their catalytic functions (Boyer et al, 1984; Hayes and Mantle, 1986; Boyer and Vessey, 1987). The GSH S-T assay was carried out at a pH of 6.5 and the GSH peroxidase assay at a pH of 7.0. Changes in pH may induce changes in the conformation of the enzyme-inhibitor complex (Vander Jagt et al, 1982; Boyer et al, 1984) which could explain to some degree the differential susceptibility to inhibition. Fig. 6.15 shows that in the GSH peroxidase assay, at a rose bengal concentration above 200 µM the percentage inhibition showed little change with increasing concentration of non-substrate ligand. At saturating concentrations of non-substrate ligand both GSH S-T Y_cY_{fetus} and Y_cY_c were able to retain approximately 40 and 50 percent of their GSH peroxidase activities respectively. Increasing the KCl concentration in the assay produced small changes in GSH peroxidase (Table 6.9) and GSH S-T (Table 6.11) inhibition of both isoenzymes.

The results of the effects of acrolein on enzyme activity (Section 6.3.6.) show that the I_{50} values for GSH peroxidase and GSH S-T inhibition of GSH S-T Y_cY_{fetus} are of a similar magnitude (Table 6.16 and Fig. 6.19). However, the GSH peroxidase activity of the Y_cY_c isoenzyme is far more susceptible to acrolein inhibition than the GSH S-T activity. The I_{50} value for GSH peroxidase inhibition is approximately half that obtained for the fetal isoenzyme (Table 6.16). In contrast, the GSH S-T activity of the fetal isoenzyme is more susceptible to inhibition than the Y_cY_c isoenzyme. This suggests that the binding of acrolein to the Y_cY_c isoenzyme, blocks the catalytic site(s) of the enzyme towards the two substrates differently. Increasing the KCl concentrations in the assays produced increases in the percentage inhibition. The effect was more marked with GSH peroxidase (Table 6.13) than GSH S-T activity (Table 6.15). This suggests that ionic factors influence the binding of acrolein to the two isoenzymes and confirms the results obtained in Chapter 5.

In summary, the results suggest that the fetal isoenzyme is far more efficient than GSH S-T Y_cY_c in catalyzing the reduction of organic peroxides. At a pH of 7, which is also the normal intracellular pH (Arieff et al, 1980), the GSH peroxidase activity of both isoenzymes can be inhibited by BSP binding. However, the GSH peroxidase activity of the fetal isoenzyme is less susceptible to ionic effects at low BSP concentrations, which may be physiologically important in utero. The fetal isoenzyme was able to retain some degree of GSH peroxidase activity despite saturating non-substrate ligand concentrations of rose bengal. This may also be physiologically important in certain toxicological and pathological states where concentrations of non-substrate ligands are high and to which the fetus may be exposed in utero. The GSH peroxidase activity of the fetal isoenzyme was far less susceptible to acrolein inhibition than the Y_cY_c isoenzyme. It is therefore attractive to suggest that should a similar situation arise in vivo, this resistance to peroxidase inhibition may play a role in preventing the fetotoxic effects of acrolein.

CHAPTER 7

CONCLUDING DISCUSSION AND DIRECTION FOR FUTURE STUDIES

In the quarter century since the first description of the GSH S-T by Booth et al (1961) new forms of this family of enzymes have been described at a rate exceeding one per year. Their central role in the metabolism of xenobiotics seems beyond question. However, several important questions still remain unanswered, including their role in protection against lipid peroxidation.

This thesis has described the identification, purification and characterization of a fetal rat liver GSH S-T isoenzyme which is absent from normal adult rat livers. The enzyme was purified using a combination of S-hexylglutathione affinity chromatography, CM-cellulose column chromatography and chromatofocusing. It is heterodimeric comprising subunits Y_c and 'Y_{fetus}' and hence referred to as GSH S-transferase Y_cY_{fetus}. The enzyme is a basic protein with an isoelectric point of approximately 8.65. It has GSH S-T activity towards a number of substrates, the most significant property being its high GSH peroxidase activity towards the model substrate cumene hydroperoxide. It is therefore attractive to suggest that this isozyme serves a specific function in protecting fetuses against the possible teratogenic effects of organic peroxides.

the GSH S-T subunits derived from fetal rat livers by affinity chromatography (Chapter 3) showed two bands with pI values of approximately 8.0 and 8.5 which were absent from adult rat livers. The band focusing at the higher pH has now been identified as GSH S-T Y_cY_{fetus}. However, the existance of the second band on isoelectric focusing suggests the presence of an additional isoenzyme specific to the fetus. The preponderance of subunits of the $Y_b/Y_{b'}$ type in the rat fetal liver suggest that this 'other' fetal isoenyme may comprise subunits of this type.

It is generally recognized that there are two critical periods of vulnerability in developmental toxicology. The first being early embryonic life and the second is in late fetal-early neonatal life (Snell, 1985). In the latter case, the developmental abnormalities that result are of a functional or metabolic nature, rather than anatomical malformations since these developmental stages are more critically involved with the functional maturation of tissues and the integration of physiological networks. Functional abnormalities are regarded as constituting the most serious hazard of developmental interference (Snell, 1985), since the consequences are insidious, often displaying latency, and in terms of dose-response relationships, occuring at lower, more environmentally relevant doses than those involved in teratogenic malformations. The isolation of GSH S-T Y_cY_{fetus} from the livers of rat fetuses which were seventeen or eighteen days old i.e. the later stages of fetal development suggest that this fetal isoenzyme may well serve an important detoxification function in the fetal rat liver.

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