Regulation Of Mouse Hepatic Glutathione S-Transferases.

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Declaration Of Originality

I declare that the work presented within this thesis, unless stated otherwise, is my own.

Catherine Dolan

June 1990

To Mum and Dad.

Abstract Of Thesis

The glutathione S-transferases (GST) are a multi-gene family of dimeric proteins which catalyse the conjugation of glutathione to a wide range of electrophilic compounds. Three classes of mouse cytosolic GST have been isolated, alpha, mu and pi, comprising Ya-, Yb- and Yf-type subunits respectively. A marked sexual dimorphism in mouse hepatic GST has been observed. The YfYf GST is the most abundant form in the male, constituting approximately 70% of total hepatic GST content. By contrast, the Yf subunit represents only a minor form in the livers of female mice. The hormonal controls which regulate the expression of the YfYf GST in mouse liver have been investigated. Testosterone, the major male sex hormone, is found to regulate the levels of Yf in mouse liver. Castration of the male leads to a decline in the levels of Yf to that observed in females. Replacement therapy with testosterone partially restores the levels of Yf. Testosterone treatment induces expression of this subunit in the female.

Growth hormone secretion from the pituitary gland differs markedly between the sexes. Androgens act to produce the male pattern of growth hormone secretion which regulates the sex-specific expression of numerous hepatic proteins. Male "little mice", specifically defective in the production of growth hormone, exhibit a feminine pattern of GST expression, despite having normal levels of testosterone. Testosterone treatment has no effect on the expression of YfYf in little mice. In contrast, growth hormone replacement therapy, administered to simulate the male-specific pattern causes an increase in the expression of the Yf subunit. These findings strongly suggest that testosterone regulates the hepatic expression of the Yf subunit indirectly through the male-specific pattern of growth hormone secretion.

The effects of the xenobiotics, phenobarbital, dexamethasone and 1,4-Bis{2-(3,5-dichloropyridyloxy)]-benzene (TCBOP) on mouse hepatic GST content have been investigated in two strains of mice, C57BL/6 and DBA/2. All three compounds were found to induce hepatic GST in both strains and sexes, predominantly affecting expression of members of the mu class. TCBOP was the most potent inducer. Hypophysectomy did not significantly affect induction of GST by these compounds.

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kd-kilodaltons

LH - luteinising hormone

lit - little

3-MC - 3-methylcholanthrene

MOPS -3-[N-morpholino]propanesulphonic acid

Mr - molecular weight

NADP-nicotinamide adenine dinucleotide phosphate

βNF - β-napthoflavone

ova - ovariectomy

PAH - polycyclic aromatic hydrocarbons

PB - phenobarbital

PCN - pregnenolone 16α-carbonitrile

PVP - polyvinylopyrrolidine

SDS - sodium dodecyl sulphate

SDS/PAGE - SDS/ polyacrylamide gel electrophoresis

sham - sham operated

TCA - trichloroacetic acid

TCDD - 2,3,7,8-tetrachlorodibenzo-p-dioxin

TCBOP - 1,4-bis [2-(3,5-dichloropyridyloxy)]-benzene

TEMED-NNN N'-tetramethylethylenediamine

tfm - testicular feminisation

TPA - 12-O-tetradecanoylphorbol-3-acetate

TRE - TPA responsive element

XRE - xenibiotic responsive element

Chapter 1 - Introduction

1.01 General Introduction

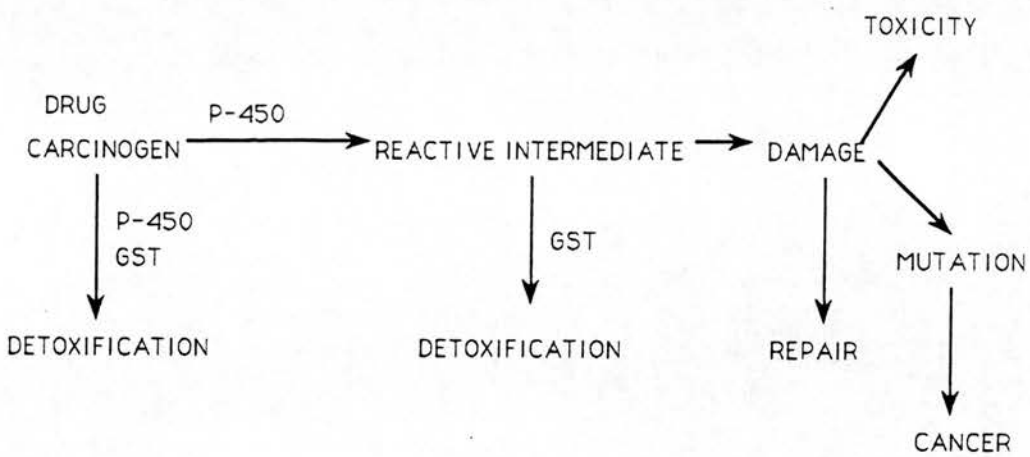
The chemical complexity of the environment today has profound significance for life at all levels in the evolutionary scale. It has been estimated that around three thousand new chemicals are synthesized each year to add to the existing hundreds of thousands of foreign compounds already in widespread use. A number of these compounds have been found to have adverse effects on biological systems and many pathological conditions such as cancer are caused by exposure to these toxic agents. How chemicals affect living organisms, how they are metabolised and how the body protects against their possible toxic effects are therefore key areas of research.[1]

Many foreign compounds are so hydrophobic that they would remain within the body indefinitely were it not for the drug-metabolising enzyme systems which are localised primarily within the liver. Two phases in the metabolism of drugs can be distinguished,[2] which are illustrated in figure 1.01. During phase I drug-metabolism, one or more polar groups, for example, a hydroxyl group, are introduced into the parent molecule. In many cases, relatively inert hydrophobic molecules are "activated" at this stage and are capable of interacting with cellular components which may result in toxicity and cell death or mutagenesis and cancer. In addition, conjugating enzymes such as the glutathione S-transferases (GST), the UDP-glucuronyltransferases or the sulphotransferases can inactivate reactive electrophilic intermediates and prevent attack on cellular macromolecules. These enzyme systems constitute the second phase of drug-metabolism. Not only are the reactive groups of the compound "neutralised" but the increased polarity of the resulting conjugate aids excretion from the body.

1- for general reviews on drug metabolism refer to Jakoby, Bend and Caldwell, 1982, Anders (1985) and Gibson and Skett, 1986).

2- Williams (1959).

Figure 1.01 Drug Metabolism - Phases I & II



Metabolism of a parent compound by the cytochrome P-450 monooxygenase family can lead to the detoxification of a toxic species. In some cases however, oxidative metabolism of a foreign compound may result in the creation of a reactive intermediate from a relatively inert starting compound. If this species is not detoxified by phase II drug metabolising enzymes, this compound can interact with cellular components and cause damage.

The cytochrome P-450-mediated monooxygenase system (cytochrome P-450, P-450) constitutes the largest group of phase I enzymes. This diverse multi-gene family of membrane-bound enzymes can interact with a wide variety of compounds both endogenous and exogenous. Although their purpose is to generate an electrophilic moiety for the phase II conjugating enzymes, metabolism of foreign compounds by cytochrome P-450s may potentiate the detrimental effects of an inert compound by activating it to a reactive toxic intermediate.

Thus, the balance within a cell between the enzymes responsible for the two phases of drug-metabolism will help determine how susceptible that cell will be to the effects of a given cytotoxic agent. Many factors can alter this equilibrium such as the genetic make-up of the individual, age, hormonal balance, nutrition, circadian rhythm, stress response and previous exposure to foreign compounds. It should also be emphasised that species differences exist in the regulation of drug-metabolising enzymes and this adds yet a further level of complexity to the situation (Eisen et al., 1983).

The regulation of the Phase I cytochrome P-450 system has received considerable attention, however, less is known of the regulatory factors which can modulate the expression of Phase II enzyme systems. This information could potentially prove invaluable to our understanding of the factors which can modulate the susceptibility of an individual to environmental toxins, and may, hopefully prove useful in future clinical strategies which aim to increase protection against environmental toxins and their detrimental side-effects by increasing the Phase II type metabolism of drugs (Wattenberg, 1978; 1985).

The work presented within this thesis is mainly concerned with the identification of endogenous factors which control the expression the glutathione S-transferases

3-for reviews see Wolf, 1986; Nebert and Gonzalez, 1987).

(GST, EC 2.5.18), an important group of phase II drug-metabolising enzymes. The model system studied was the mouse and the investigation concentrated solely on the liver, which is the major site for detoxification within the body. The effect of exogenous compounds on this system was also studied.

1.02 Glutathione conjugation - a major step in the mercapturic acid pathway

Glutathione, the major non-protein thiol species within the cell, is universally present in all aerobic forms of life (for review, see Arias and Jakoby, 1976). It exists in either the reduced form (GSH) or in the oxidised state (GSSG). Reduced glutathione is a tripeptide consisting of the amino acids γ-glutamic acid, cysteine and glycine. The γ-glutamyl linkage is thought to protect against degradation. The structure is shown in figure 1.02. Although the GSH concentration varies between tissues, this species is relatively abundant and normally exists in the reduced form (GSH), however it can readily undergo conversion to the oxidised form (figure 1.02). At physiological pH, GSH has a net negative charge and is highly water soluble. The nucleophilic thiol group present on the cysteine moiety confers reactivity towards electrophiles (Boyland and Chasseaud, 1969).

The mercapturic acid pathway is a major route in the biotransformation and excretion of foreign compounds. The conjugation of GSH to reactive electrophilic intermediates constitutes the first step in this pathway, which is depicted in figure 1.03. Booth *et al.* (1961) described an activity in the liver which catalysed this conjugation reaction and subsequently the enzymes which catalyse these reactions have been known as the glutathione S-transferases. The GSH conjugate once formed can be excreted directly or can undergo further metabolism to yield the corresponding mercapturic acid (Habig *et al.*, 1974).

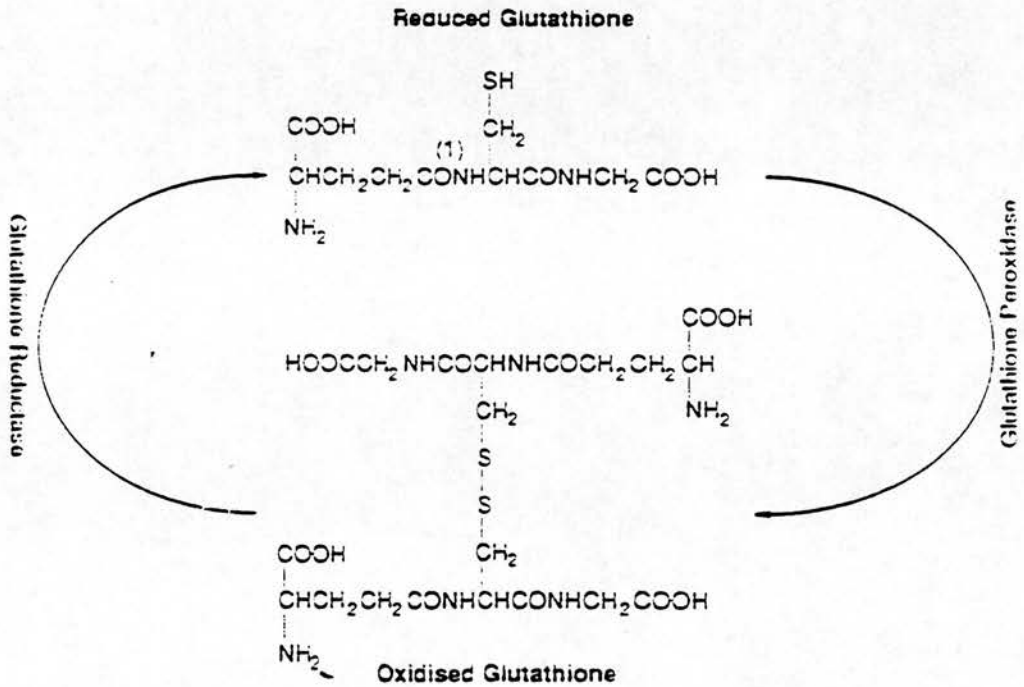


Figure 1.02 - Structure Of Glutathione

The inter-relationship between reduced glutathione and oxidised glutathione (glutathione disulphide; GSSG) and their chemical structures.

[(1) - γ-glutamyl peptide link]

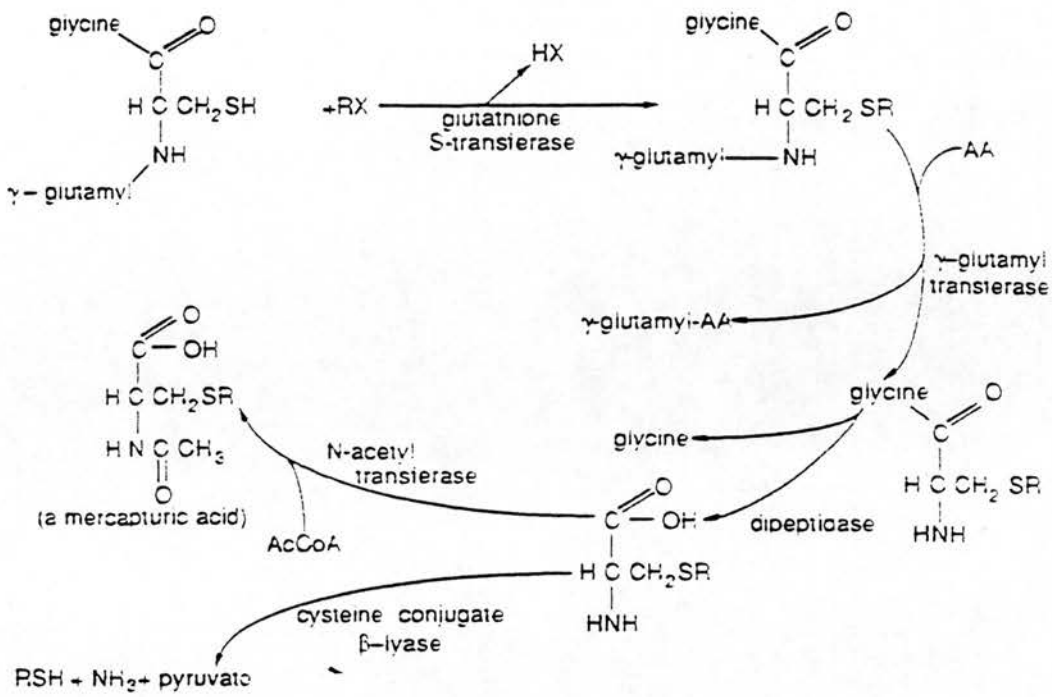


Figure 1.03 Mercapturic Acid Pathway.

Conjugation of an electrophilic compound (RX) with glutathione is catalysed by the glutathione S-transferases. The conjugate may be directly excreted into bile. Alternatively, the glutathione conjugate may undergo further metabolism. In the kidney, the enzyme γ-glutamyl transferase catalyses the transfer of the γ-glutamyl moiety to neutral amino acids (AA) or peptides. After which, cysteinyl-glycinase (dipeptidase) catalyses the hydrolysis of the cysteinyl-glycine peptide bond, with the release of glycine. N-acetlylation of the cysteinly derivative catalysed by the enzyme N-acetly transferase yields the mercapturic acid. Alternatively, the cysteine conjugate may be cleaved to a mercaptan by the elimination of pyruvate and ammonia.

After conjugation of the foreign compound with GSH, the glutamic acid residue is transferred by the microsomal enzyme, γ-glutamyltransferase to neutral amino acid or peptide acceptors. Following removal of the glutamic acid, the glycine residue may also be lost from the remaining cysteinylglycine conjugate. Finally, the cysteine derivative is acetylated to yield the mercapturic acid, a classical excretion product of xenobiotics. An alternative metabolic route leads to the formation of the mercaptan through cleavage of the cysteine conjugate and the elimination of pyruvate and ammonia.

1.03 Nomenclature and classification of GST

Initial reports by Booth *et al.* (1961) demonstrated the presence in rat liver cytosol of an enzymatic activity which catalysed the formation of GSH conjugates with a variety of compounds. Soon after it was recognised that several distinct enzymatic activities exist (Johnson *et al.*, 1963). Early attempts to classify these enzymes were based on their activity towards certain classes of substrate hence the terms aryltransferase, epoxide transferase, alkytransferase, aralkytransferase and alkenetransferase. However, as purification schemes were developed, it became clear that individual forms each exhibit a broad, overlapping substrate specificity that were not restricted towards a certain class of chemical. Thus, the early substrate-based nomenclature was rendered obsolete and replaced by enzyme designations which reflected the chromatographic properties of GST (Fjellstedt *et al.*, 1973; Habig *et al.*, 1974; Pabst *et al.*, 1973).

More recently, the chromatography-based designations have been superseded and the two major nomenclatures in use today reflect the quaternary structure of the glutathione S-transferase isoenzymes. Cytosolic GST forms consist of two subunits.

Homo- and heterodimeric combinations of individual subunits give rise to a multiplicity of GST isoenzymes, each with a unique spectrum of catalytic activity (Hayes *et al.*, 1981; Mannervik and Jensson, 1982). In the rat, which has been the most fully characterised system to date, many distinct subunits have been identified and characterised. The nomenclature system which has been employed throughout the course of this thesis is based on the work of Bass *et al.* (1977), where subunits were designated according to their mobility on SDS/PAGE. The subunit forms originally observed in rat liver preparations were named Ya, Yb and Yc in order of their decreasing mobility on SDS/PAGE. This nomenclature has since been extended to incorporate other GST subunits which have since been identified (Hayes,1983; Hayes and Mantle, 1986a,b; Ishikawa *et al.*, 1987). The relative mobilities of GST subunits on SDS/PAGE using a 12% polyacrylamide gel (C_{Bis} 2.6%) are Yf > Yk > Ya > Yn > Yb > Yc (Hayes, 1986; Hayes and Mantle, 1986)

In the other major nomenclature system proposed by Jakoby *et al.* (1984), individual subunits have been assigned Arabic numerals. The relationship between these current nomenclatures is given in table 1.01, this table has also been extended to include older nomenclatures based on purification schemes. In section 1.07, the rat GST forms are related to those forms found in the mouse and the nomenclature has been further extended to encompass this species.

1.04 The glutathione S-transferases - a multi-gene family of proteins

Cytosolic glutathione S-transferases can be divided into three distinct families on the basis of their structural, immunochemical and catalytic activities (Mannervik, Mannervik and Danielson,
1985, 1988). These properties ultimately depend on the primary sequence of the

Table 1.01 GST Nomenclatures (for rat)

Class	GST*	1	2	3	4
alpha	YaYa	1 - 1		Ligandin	
	YaYc	1 - 2	B	B	
	YaYc	2 - 2	AA		
	YkYk	8 - 8		K	
mu	Yb1Yb1	3 - 3	A		
	Yb1Yb2	3 - 4	C		
	Yb2Yb2	4 - 4			
	YnYn	6 - 6		N	
	Yb1Yn	3 - 6		P	
	Yb2Yn	4 - 6		S	
pi	YfYf	7 - 7			P
ND		5 - 5	E		

ND - no designation

* Nomenclature utilised throught the course of this thesis.

1 - Jakoby *et al.* (1984)

2 - Fjellstedt *et al.* (1973), Habig *et al.* (1974, 1976)

3 - Hayes *et al.* (1979)

Hayes (1984, 1986)

4 - Kitihara *et al.* (1984)

protein. Within gene families a high degree of primary sequence homology exists, greater than 80%. Individual subunits from the same gene family display a high degree of cross-reactivity at both the protein and nucleic acid levels. The primary sequence homology between families is poor (less than 30%). Heterodimeric forms can arise from the hybridisation of different subunit types, however only subunits within the same gene family can form binary combinations (Hayes *et al.*, 1981; Mannervik and Jensson, 1982; Hayes *et al.*, 1984). Between families no cross-reactivity is observed at either the protein or nucleic acid level. The three cytosolic gene families have been designated alpha, mu and pi (Mannervik *et al.*, 1985) or Groups I, II, III (Hayes and Mantle, 1986b). The alpha class family consists of the Ya, Yc and Yk subunits, the mu class GST contain Yb- and Yn-type subunits and the YfYf GST constitutes the pi class GST. Members of these families are listed in table 1.02. The microsomal GST (Morgenstern *et al.*, 1979, 1980, 1982) constitutes a fourth gene family, which was not studied during this thesis.

Class Alpha

The alpha class comprises Ya-, Yc- and Yk-type subunits. The primary amino acid sequence for both Ya and Yc have been deduced from full-length cDNA clones (Pickett *et al.*, 1984; Telakowski-Hopkins *et al.*, 1985). The overall nucleotide sequence identity between Ya and Yc is 66%. Within the protein coding region, this identity is higher (75%). The overall amino acid sequence identity between the Ya and Yc subunits is 68%.

Southern blot analysis of rat genomic DNA using specific probes to the 5' and 3' regions of the rat Ya and Yc genes also indicate the presence of multiple genes encoding both these subunits. It has been estimated that at least five Ya genes and two

Table 1.02 GST Gene Family Members

Gene Family	Subunit	M r	Homology* (within the family)
Alpha (I)	Ya	25,500	68%
	Yc	27,500	
	Yk	25,000	
Mu (II)	Yb_1	26,300	
	Yb_2	26,300	> 80%
	Yn	26,000	
Pi (III)	Yf	24,800	80%**
	FAEE sythetase	26,000	

* Homology at the amino acid level.

** N-terminal amino acid homology, (Bora *et al.*, 1989)

Yc genes are to be found within the rat genome (Rothkopf *et al.*, 1986). cDNA clones encoding the rat Ya subunit have been isolated by various laboratories, these clones fall into two distinct types which differ by 8 amino acids out of 222 residues. The "prototype" clones for the these types of gene are pGTB38 isolated by Pickett *et al.* (1984) and pGTR261 isolated by Lai *et al.* (1984). A third cDNA isolated again by Pickett and his colleagues is identical in the protein region to the cDNA clone described by Lai *et al.* (1984), however, its 3' untranslated region is extended in length and contains a type 2 alu repetitive element (Rothkopf *et al.*, 1986).

The presence of multiple genes in this family was also confirmed by the isolation of four unique genomic fragments from a rat genomic library (Rothkopf *et al.*, 1986). One of the isolates λ GTB45-15 was found to encode a Ya subunit. This gene spanning 10kb in length comprises 7 exons separated by 6 introns. To date, this has been the only report of an alpha class genomic clone (Telakowski- Hopkins *et al.*, 1986).

Evidence also exists at the protein level for microheterogeneity within subunit types. Sheehan and Mantle (1984) provided evidence for two forms of Ya subunit, which appear structurally very similar but are quite distinct in their kinetic parameters and their inducibility with phenobarbital.

More recently, Hayes *et al.* (1990) have used reverse-phase h.p.l.c. to resolve two separate Ya-type subunits from rat liver and demonstrated their distinct genetic origins by amino acid sequencing. The subunits were designated Ya_1 and Ya_2 and their primary structures suggest that they are encoded by pGTR261 and pGTB38, respectively. Interestingly, Ya_2 is preferentially induced in aflatoxin B_1-induced nodule-bearing livers but the relationship between Ya_1 and Ya_2 and the two forms of Ya described by Sheehan and Mantle (1984) is unclear.

Amino acid composition and immunochemical data (Hayes, 1986; Hayes and Mantle, 1986) suggest that the Yk subunit shares sequence homology with the Ya and Yc subunits, it has therefore been placed within this group. However, a closer degree of homology exists between Ya and Yc than these subunits share with Yk and Alin *et al.* (1989) have reported that Yk possesses 57-60% positional identity with Ya and Yc. As of yet no rigid criteria exist to define what constitutes a gene family. The degree of primary sequence homology which is "acceptable" between distinct forms within a family has not been defined. It is therefore uncertain as to whether the Yk subunit should be classed within the alpha gene family or whether it should be classed as a member of a separate but related subfamily.

Class Mu

The mu class family comprises Yb- and Yn-type subunits. cDNA clones encoding the Yb_1 and Yb_2 subunits have been isolated by Ding *et al.* (1985a, 1986) and a cDNA encoding the Yn subunit (also known as Yb_3) was cloned by Abramovitz and Listowsky (1987). Greater than 80% homology exists between these GST subunits at both the DNA and protein level. Southern blot analysis again indicates the presence of multiple genes (Lai *et al.*, 1986). Genomic sequences for the Yb_1 and Yb_2 clones have been obtained but as of yet no genomic clone has been described for the Yn subunit (Tu *et al.*, 1987). A genomic clone has been isolated by Lai *et al.* (1988) which encodes a novel mu class enzyme termed Yb_4, which is homologous to, but not identical to, other mu class GST forms.

At the protein level, Yb_1Yb_2 dimers have been found to exist in vivo (Hayes, 1983). In vitro hybridisation studies demonstrated that both Yb subunits can dimerise with the Yn subunit. Transferase P and transferase S isolated from rat liver correspond to

the Yb_1Yn and Yb_2Yn heterodimers respectively (Hayes and Chalmers, 1983; Hayes, 1984).

Class Pi

Until recently, evidence suggested that the pi class GST family contained only the member, the Yf subunit. Although multiple bands appear on southern blotting with a cDNA clone encoding the rat Yf subunit, Okuda *et al.* (1987) have reported that the Yf GST was the product of a unique gene locus. All other potential clones isolated were found to be non-functional pseudogenes. Recently, however, the major FAEE synthetase, isolated from human heart was found to be 72% identical to the N-terminal sequence of the rat Yf subunit and 82% homologous to GST YfYf isolated from human heart (Bora *et al.*, 1989). Subunits within other families, as discussed above, display similar levels of homology.

Catalytic activities are also interchangeable, in that FAEE synthetase can catalyse the conjugation of GSH with CDNB and the GST isolated from bovine liver was active in the formation of FAEE. Further reports on this enzyme and its relationship to the pi class GST are awaited with considerable interest as this family might be more complex than was originally thought.

Further Cytosolic GST Classes?

Subunit 5-5, which as yet has received no designation in the Y nomenclature, may be related to the Yb\Yn family, although this is not as yet certain (Meyer *et al.*, 1984). Further molecular characterisation of this subunit form will need to be performed before it is clear whether it is a member of the alpha, mu, pi or other family of GST.

1.05 Catalysis

The glutathione S-transferases catalyse the conjugation of GSH to a wide variety of electrophilic compounds. Many GST substrates are detrimental to the cell due to their ability to interact with cellular macromolecular components causing cellular toxicity or in the case of DNA, mutagenesis. A comprehensive review of the wide GST substrate specificities towards carcinogenic compounds is given by Chasseaud (1979).

Although cytosolic GST forms are dimeric, studies on the kinetics of GST catalysis indicate that each subunit is catalytically independent. No allosteric or cooperative effects have been described (Danielson and Mannervik, 1985). Each individual isoenzyme has a unique spectrum of catalytic activity, the additive effects of individual subunit combinations, i.e., the kinetic properties of the heterodimer can be predicted from those of the corresponding homodimers (Yalcin *et al.*, 1983; Tahir and Mannervik, 1986).

In the case of compounds such as CDNB, the substrate specificities of individual GST isoenzymes are broadly overlapping, although different forms have a distinct spectrum of specific substrates activities. The compound CDNB is used as the "general" GST substrate, although some forms of GST have a very low specific activity towards this substrate (Clark *et al.*, 1973).

Glutathione conjugation to certain substrates is restricted to specific enzymatic forms (Mannervik and Jensson, 1982). These "model" substrates can be used to distinguish between individual subunit forms within a gene family, which can be particularly useful when members cannot be distinguished on the basis of molecular

weight or immunochemistry. Two substrates which are particularly useful are DCNB and *trans*-4-phenyl-3-butene-2-one which are marker substrates for the Yb_1 and Yb_2 subunit forms.

Two distinct GSH peroxidase activities can be can be distinguished in the liver, a Selenium-dependent GSH peroxidase and a Selenium-independent GSH peroxidase. This Se-independent activity is contributed by the alpha class GST, particularly the Yc subunit (Lawrence and Burk, 1976 ; Carmagol *et al.*, 1983). Both activities can be distinguished enzymatically by the use of H_2O_2, which is specific for the Se-dependent GSH peroxidase activity, whereas both forms are active with the organic hydroperoxide, cumene hydroperoxide. This substrate is used as a marker substrate for alpha class Ya, Yc and Yk subunits in the rat.

Table 1.03a lists the model substrates for specific GST subunits. Table 1.03b gives the specific activities for these substrates with a range of GST isoenzyme forms.

1.06 Endogenous Compounds as GST substrates.

The emphasis with regards to GST substrates has concentrated on their activity towards xenobiotic compounds either as tools to discriminate between individual isoenzyme forms or in their ability to detoxify harmful compounds. It should be noted that in addition to their role in xenobiotic detoxification, increasing evidence indicates that the GST family might also play an important part in normal cellular metabolic processes, particularly in protection against the products of the oxidative metabolism of endogenous compounds. In addition, these enzymes are probably also involved in normal biosynthetic pathways within the cell. Potential natural substrates for the GST are given below.

Table 1.03 a

Characteristic substrates for rat cytosolic GST subunits

Subunit	Characteristic substrate
Ya	Δ^5-androstene-3,17-dione cumene hydroperoxide
Yc	cumene hydroperoxide
Yb_1	1,2-dichloro-4-nitrobenzene
Yb_2	*trans*-4-phenyl-3-buten-2-one
Yf	ethacrynic acid
Yn	1-chloro-2,4-dinitrobenzene
Yk	4-hydroxyalkenals
5	1,2-epoxy-3-(p-nitrophenoxy) propane

Table 1.03b

Specific activities (μmoles/min/mg) of rat glutathione S-transferases

Substrate	Class	Alpha			Mu			Pi
	Subunit	Ya	Yc	Yk	Yb_1	Yb_2	Yn	Yf
1-chloro-2,4-dinitrobenzene		50	17	10	58	17	190	24
1,2-dichloro-4-nitrobenzene		<0.04	<0.04	0.12	5.3	0.18	2.85	0.048
ethacrynic acid		0.08	1.24	7.0	0.08	0.62	0.057	3.84
trans-4-phenyl-3-buten-2-one		<0.004	<0.004	0.10	0.05	1.18	0.019	0.22
cumene hydroperoxide		3.1	7.9	1.1	0.35	0.72	0.19	0.048
Δ^5-androstene-3,17-dione		4.2	0.36	--	0.02	0.002	--	--

taken from Mannervik and Danielson (1988).

As has been previously referred to in section 1.05, GST isoenzyme forms are found to have activity towards the organic peroxide, cumene hydroperoxide, which is used as a model substrate for the alpha class GST forms, particularly the Yc subunit (Lawrence and Burk 1976, Mannervik, 1985). Linoleate and arachidonate hydroperoxides have also been found to have good activity with GST forms, the uncharacterised form 5-5 having the highest activity with these substrates (Ketterer *et al.*, 1987). Other compounds, generated in the process of lipid peroxidation are found to act as substrates for GST. Arachidonic acid gives rise to several epoxide derivatives which are substrates for GST (Spearman *et al.*, 1985). Alkenes, yet another by product of lipid peroxidation are also reactive with GST. The 4-hydroxyalkenals, major products of lipid peroxidation, are good substrates for GST, the Yk subunit (form 8-8) being exceptionally active with these compounds. The Ya and Yb_2 subunits also have high activity with these substrates but several fold less than the Yk. (Alin *et al.*, 1985, Danielson *et al.*, 1987).

Cholesterol-α-oxide is yet another product of lipid peroxidation, a possible carcinogen, which has been found to be a substrate for GST. The Ya subunit was found to have highest activity with this compound (Watabe *et al.*, 1979, Meyer and Ketterer, 1982) .

Thus one of the major endogenous roles for the glutathione S-transferases might be in cellular protection against lipid peroxidation. Indeed, Tan *et al.* (1984) have shown that the Se-dependent GSH peroxidase and alpha class GST inhibit lipid peroxidation in vitro.

DNA hydroperoxides have also been shown conjugated to GSH. Peroxidised DNA has been shown to serve as a substrate for rat GST, highest activity was obtained with the

mu class (Tan *et al.*, 1986; Ketterer *et al.*, 1987). This is especially interesting in the light of the report by Bennet *et al.* (1986) in which mu class GST was located in rat liver nuclei. Thus, GST may play a role in the protection of DNA, possibly preventing peroxidative damage to the cell's nucleic acid.

Quinones may represent a class of reactive compounds which may be detoxified by GST. Dopaquinone has been found conjugated to GSH in human malignant melanoma (Agrup *et al.*, 1977). Reactive intermediates of oestradiol metabolism may also serve as substrates for GST (Jellinck *et al.*, 1967; Marks and Hecker, 1969).

In addition to their general role in protection against reactive cellular intermediates, these enzymes might also play a normal part in the biosynthesis of biologically active molecules. The leukotrienes and prostaglandins, signalling molecules derived from arachidonic acid, are substrates for GST (Christ-Hazelhof *et al.*, 1976; Tsuchida *et al.*, 1987). GST isoenzyme forms have been reported to exhibit prostaglandin isomerase activity including the Ya subunit which has also been shown to have steroid isomerase activity with Δ^5-androstene-3,17-dione. (Benson *et al.*, 1977; Meyer and Ketterer, 1987) Certain of the major leukotriene derivatives with strong physiological activity originate from conjugation with GSH. GST forms have been found to catalyse the conversion of leukotriene A4 to leukotriene C4, the class mu Yb_2 subunit being most active (Mannervik *et al.*, 1984). Thus GST may play a role in the complex biological pathways involved in the synthesis of steroids, prostaglandins and leukotrienes.

1.07 Mouse Hepatic Glutathione S-Transferases.

Mouse glutathione S-transferases have been investigated by several laboratories (Lee *et al.*, 1981; Pearson *et al.*, 1983; Aigius and Gidari, 1985; Hatayama *et al.*, 1986; Warholm *et al.*, 1986 ; Hayes *et al.*, 1987; McLellan and Hayes, 1987; 1989; Benson *et al.*, 1989). Early purification studies yielded information that was unclear and inconsistent. The physical properties of the enzymes isolated by various laboratories differed making it difficult to determine the number of murine GST and establish a helpful nomenclature.

Lee *et al.* (1981) isolated from DBA/2 mice (sex not specified) three major cytosolic forms termed F1, F2 and F3. Enzymes F1 and F2 both had Mr values of 22,000 and were reported to be charged isomers with pI values of 6.5 and 8.2 respectively. F3 was found to have a Mr of 27,000 and a pI value of 8.8.

The hepatic GST content of CD-1 mice was investigated by Pearson *et al.* (1983) and Agius and Gidari (1985). Both groups described the purification of two enzyme forms. Pearson and his colleagues isolated from female mice treated with butylated hydroxyanisole (BHA) both a major GST form termed GT 8.7 and a minor cytosolic GST form, GT 9.3, named on the basis of their isoelectric points. Both forms had Mr values of 24,000, were immunologically cross-reactive and had a high degree of N-terminal sequence homology.

Agius and Gidari (1985) described the isolation of two homodimeric forms from the liver of female CD-1 mice treated with streptozotocin, a drug used to induce diabetes in animal models. These enzyme forms had Mr values of 27,500 and 28,000 with pI values of 9.2 and 8.2 respectively. It was thought that these forms corresponded to

the GT 8.7 and GT 9.3 forms described by Pearson *et al.* (1983). Discrepancies were thought to be due to technical factors.

More recently, data by a number of workers (Hatayama *et al.*,1986; Warholm *et al.*, 1986; Hayes *et al.*, 1987; McLellan and Hayes, 1987; Benson *et al.*, 1989) demonstrate that in mouse liver, GST isoenzyme forms exist which have homology to the rat species described and can be divided into the three gene classes, alpha, mu and pi. The properties of the enzyme forms isolated and characterised by various workers are described in table 1.04

Warholm *et al.* (1986) purified three enzyme forms from the livers of male NMR1 mice. These forms, referred to as MI, MII and MIII appeared to be dimers of identical subunits. Transferase MI was assigned to the alpha class whereas transferases MII and MIII were placed in the pi and mu classes respectively. The specific activities of the GST isoenzymes isolated from mouse liver with a range of GST substrates are shown in table 1.05. Activities of the three mouse GST forms towards the model substrates listed in table 1.03 (a,b) compare closely with those of the rat (refer to table 1.03b).

Hayes *et al.*(1987), investigating the hepatic GST content of male LACA mice, reported similar findings. Three GST forms were isolated which were found to be homodimeric enzymes comprising Yf-(Mr 24,500), Ya-(Mr 26,000) and Yb (Mr 27,000) type subunits.

Interstrain variation in GST content was proposed to account for the lack of consistency in early work. Hatayama *et al.* (1986) and McLellan and Hayes (1987)

Table 1.04 Characteristics of mouse hepatic cytosolic GST isoenzymes isolated by different laboratories.

Reference	Tissue Source	GST designation	Subunit Mr	pI
Lee *et al.* (1981)	DBA/2	F1	22,000	6.5
		F2	22,000	8.2
		F3	25,000	8.8
Pearson *et al.* (1983)	CD-1	GT 8.7	24,000	8.7
		GT 9.3	24,000	9.3
Agius & Gidari (1985)	CD-1	C/S2	28,000	8.2
		C/S3	27,500	9.2
Warholm *et al.** (1986)	NMR-1	MI	25,000	9.7
		MII	23,000	8.7
		MIII	26,500	8.5
Hayes & Mantle* (1986)	LACA	YfYf	24,800	8.6
Hayes *et al.* (1987)		YfYe	24,000 + 24,800	ND
		YaYa	25,800	> 9.2
		YbYb	26,400	7.8 - 8.2
Hatayama *et al** (1986)	BALB/C	ml (Ya)	27,000	> 10
	C57BL/6	m2 (Yf)	26,000	9.3
	CD-1	m3 (Yb)	28,000	9.1
	CH3/He			
	DBA			
McLellan & Hayes* (1987)	DBA/2	YfYf	24,800	8.6
	CH3/He	YaYa	25,800	>9.2
	C57BL/6	YbYb	26,400	7.8 - 8.2

Abbreviations : ND - not determined
* GST preparations from untreated, normal mouse liver.

Table 1.05

Specific activities (μmol/min/mg) of constitutively expressed mouse hepatic glutathione S-transferases

Class:	alpha	mu	pi
Substrate			
1-chloro-2,4-dinitrobenzene	19	148	119
1,2-dichloro-4-nitrobenzene	0.062	4.4	0.14
ethacrynic acid	0.025	0.12	1.4
trans-4-phenyl-3-buten-2-one	0.009	0.004	0.013
cumene hydroperoxide	11.6	0.11	0.14
Δ^5-androstene-3,17-dione	0.035	0.043	0.14

Taken from Mannervik and Danielson (1988).

investigated the hepatic content of several mouse strains to determine if interstrain differences in hepatic GST content did occur. GST forms homologous to the three rat cytosolic gene families were isolated and characterised. Both groups however found no evidence of strain differences in hepatic GST content. However, a marked sex difference in the expression of the pi class GST was observed. In the male mouse liver this subunit is the major GST form, constituting approximately 70% of the total GST content. In contrast, this subunit constitutes only a minor form in the female mouse liver. This significant difference in the expression of the Yf subunit between the sexes might account to some extent for the differing results in the early work.

Many purification schemes isolated hepatic GST from mice which had been treated with compounds which can induce hepatic GST. This would have led to some confusion as certain GST forms are not present in significant quantities in uninduced liver. Pearson *et al.* (1983) isolated from BHA-treated mice GT 8.7 and 9.3, both proteins are members of the mu class. GT 8.7 was found to be expressed in normal liver, however, GT 9.3 expression was found to be very low in mice fed normal diets.

McLellan and Hayes (1989) described the induction of a novel alpha class GST subunit in DBA/2 mice after treatment with BHA. This subunit, termed Ya_1, on the basis of immunological and physical properties was more closely related to the rat Ya subunit than the constitutively expressed murine Ya subunit (known as Ya_3). In the uninduced liver Ya_1 was barely detectable, after treatment with BHA, this subunit was induced more than 20-fold.

Recently, Benson *et al.* (1989) reported the purification from CD-1 mice of enzyme forms (GT8.7, GT8.8a, GT8.8b, GT9.0, GT10.3 and GT10.6) from all three GST gene

families. Forms GT 8.7 and 9.3 were identified and confirmed as mu class. Other forms were also noted which differed slightly in the pI values from GT 8.7 (GT 8.8a, GT8.8b). Evidence presented indicated that these forms were essentially identical to GT 8.7 and could be ascribed to the mu class. Form GT 9.0 was classified as pi class. On the basis of electrophoretic mobility during SDS/PAGE (Mr not quoted), a blocked N-terminus and enzymatic activity, GT 10.6 was assigned to the alpha class, although a high degree of disparity was observed between the amino acid composition of this species and the alpha class clone pGT41 (Pearson *et al.*,1988). Another form, GT 10.3 was described which again was thought to belong to the alpha class. This species when subject to h.p.l.c. split into two peaks; one peak had a Mr similar to GT 10.6 and the other was 1kd smaller. GT 10.6 was not significantly affected by BHA treatment, GT 10.3 however was barely detectable in the untreated liver but induced after treatment. It is possible that these forms correspond to the alpha class GST subunits characterised by McLellan and Hayes (1987,1989). GT 10.6 might correspond to the constitutively expressed Ya_3 subunit and GT10.3 might correspond to the Ya_1 subunit.

GT 8.7 was found to be the major GST form induced on treatment with BHA. Interestingly, however, the Yf subunit was also induced in the female mouse but not in the male, where this GST is normally the most abundant form. Possibly the Yf GST in the male is expressed at the highest level possible and can not be induced to any further extent.

1.08 Molecular analysis of mouse GST.

The first cDNA clone encoding a mouse GST was isolated by Pearson *et al.* (1983). The clone, pGT55, was found to be identical to the protein GT 9.3 and highly

homologous to GT 8.7 (85% amino acid identity) in the N-terminal region of the proteins for which sequence information was available. Later, a cDNA clone encoding the GT 8.7 protein was reported by Pearson *et al.* (1988). Both clones are highly homologous to the mu class GST subunits from rat. GT 8.7 is very closely related to the rat Yb_1 subunit sharing 92% amino acid sequence identity.

Townsend *et al.* (1989) isolated from mouse L929 cells two mu class cDNA clones, pGT10 and pGT2 which encoded proteins termed mGTmu1 and mGTmu2. Sequence analysis showed mGTmu1 to be completely identical except for one amino acid difference to GT 8.7, this form was more closely related to the rat Yb_1 subunit than any mouse mu class GST enzymes isolated to date. The second clone mGTmu2 was found to be very closely related to the rat Yb_2 subunit, sharing 95% sequence identity at the amino acid level. The cDNA isolated by Pearson *et al.*(1983), pGT55 was highly homologous to the deduced amino acid sequence for the fourth rat mu class GST clone described (Lai *et al.*, 1988), which was isolated from a genomic library. Sequence identity was very high with replacement of only 5% of amino acid residues. Messenger RNA encoding the mGTmu1 was found in high levels in normal liver, whereas mGTmu2 was not detectable.

Southern blot analysis of mouse DNA using a cDNA clone encoding the rat Ya subunit gives a complex multi-band pattern indicative of a multi-gene family. At least four genes were estimated to be present in the mouse genome. All hybridising bands were located to mouse chromosome 9 by the use of somatic cell hybrids and they appeared to be clustered (Czosnek *et al.*,1984).

Daniel *et al.* (1987) reported the isolation of a genomic clone encoding a mouse Ya subunit which spanned 11kb of DNA and contained 7 exons. A comparison of the

deduced amino acid sequence from the mouse genomic clone with the rat alpha class Ya_1 subunit showed a high degree of conservation with 10 amino acid differences between species.

Pearson *et al.*(1988) described the isolation of a mouse Ya clone pGT41 which is very similar although not identical to the coding sequence for the genomic clone isolated by Daniel *et al* (1987). Nucleotide sequence identity was 97%, with 96% homology at the amino acid level. This gene is highly homologous to the rat Ya subunit sharing 95% amino acid identity, homology to the Yc subunit was reported at 72%. pGT41 and the mouse genomic clone isolated by Daniel could represent two different species of mouse Ya subunit analogous to the two classes of Ya cDNA clones which have been isolated in the rat.

Northern blot analysis showed the mRNA species hybridising to pGT41 is present at very low levels in the uninduced liver. Upon BHA treatment however, a 50-fold increase in the level of this mRNA has been observed (Pearson *et al.*, 1988). The BHA-inducible Ya subunit (Ya_1) described by McLellan and Hayes (1989) is also present at very low levels in the untreated mouse being induced more than 20-fold in the BHA-treated animal. This subunit would also appear to be more closely related to the rat Ya than the constitutive Ya subunit (Ya_3) expressed in mouse liver, which is not significantly affected by BHA-induction. It is therefore probable that the clones isolated by Pearson *et al.* (1988) and Daniel *et al.* (1987) encode Ya_1-type subunits rather than Ya_3. The cloning strategies employed in both cases used cDNA clones encoding the rat Ya subunit to probe for mouse alpha class genes. Daniel *et al.* (1987) used a mouse genomic library and Pearson *et al.* (1988) used a cDNA library from BHA-induced mouse liver. The more closely related GST form would therefore be preferentially detected on screening these libraries. Protein sequence

data on both mouse alpha class GST forms will add further light to this situation. Table 1.06 lists the mouse hepatic subunits characterised to date with their probable rat equivalents, homologies where known, are quoted.

The mouse would appear at first hand to be a simpler system than the rat in respect to GST expression. The evidence presented indicates that in normal liver three homodimeric GST forms are present Ya_3, Yb_1 and Yf, in contrast to the rat where 7 GST subunit forms have been isolated and characterised. Other forms are either not expressed or found in very low levels. Upon treatment with GST inducing agents not only is the induction noted of certain of the subunits normally expressed in the liver, but other forms of GST are induced as well. At least one other alpha class GST is induced after BHA treatment, together with mu class GST Yb_2. Doubtless other forms are present in the liver or expressed in extrahepatic tissue which have as yet to be characterised. Information at both the molecular and protein level will provide a more thorough basis for the classification of the forms which have already been characterised together with those forms which have as yet to be noted.

1.09 Regulation of Glutathione S-Transferases.

The regulatory factors which determine the expression of the glutathione S-transferases are complex as is the enzyme system itself. Control is exerted at many levels. GST expression is regulated during development, tissue specificity in expression occurs and sexual dimorphism in the level of GST occur. However, little information is available on the underlying mechanisms which govern GST expression within the cell. The endogenous factors which regulate these processes remain largely unidentified.

Table 1.06 Mouse GST subunits related to the rat GST subunits

Class	Mouse subunit	Mr (Subunit)	Equivalent rat subunit	Sequence Identity (nucleotide)
Alpha	Ya1*	25,600	Ya	ND
	Ya3	25,800		ND
Mu	Yb1 (GT8.7)	25,801	Yb_1	93%**
	Yb2 (mGTmu2)	25,548	Yb_2	95%**
	Yb3 (GT9.3)	24,000	Yb_4	95%**
Pi	Yf	24,800	Yf	ND

Abbreviations : ND - not determined
* data obtained from McLellan & Hayes (1989)
** data obtained from Townsend *et al.*,(1989) all other data obtained from McLellan & Hayes (1987).

Most tissues contain GST in relatively abundant amounts although highest levels are found in the liver, the major site of detoxification in the body (Hayes and Mantle, 1986). In the adult rat, a marked tissue-specificity in the expression of GST has been noted by several workers (Hayes and Mantle, 1986; Tu *et al.*, 1987). The Yf containing GST provides a very notable example of tissue-specific regulation. In the liver and testis this GST form is present in barely detectable amounts, whereas Yf constitutes a major form in most other tissues, particularly the spleen and colon (Hayes and Mantle, 1986). This tissue-specificity in expression is also reflected at the mRNA level (Tu *et al.*, 1987).

Within a tissue, GST expression might not be uniformly distributed. Redick *et al.* (1982) using immunohistochemistry demonstrated that alpha and mu class GST, together with transferase E, are expressed in the parenchymal cells throughout the liver. However their concentration was highest in the centrilobular region. In the bile duct epithelium, transferase E and class mu GST gave positive staining but no alpha class GST was detected. Again the factors which control the differential expression of GST within a tissue are not known. Immunohistochemistry will prove to be an invaluable tool when investigating GST regulation. It will clearly be important to establish not only the tissue-specificity in the expression of this enzyme system but also the expression of GST within individual cell-types. This might possibly provide information on the biological reasons for marked differences in expression between tissues. As of yet these differences are not known but could possibly be related to the specialised requirements of a given cell-type.

Major differences in hepatic GST expression have been observed between the sexes in both the rat and mouse. In rat liver, levels of the Yb_1 subunit are several-fold

higher in male liver than in the female. Levels of the Ya subunit, in contrast are higher in the female than in the male (Hales and Neims 1976, Lamartiniere, 1981 Igarashi *et al.*, 1983,1985, 1987).

In mice, a marked difference in the expression of GST subunits has been observed in the livers of males and females (Hatayama *et al.*, 1986, McLellan and Hayes, 1987). In contrast to the rat, where YfYf is barely detectable in the liver, the Yf subunit in male mouse constitutes over 70% of total hepatic GST. In the female however, this subunit is a minor form expressed at levels approximately 10-fold lower than in the male.

In both species, developmental regulation of liver GST expression occurs. In both mice and rats, levels of hepatic GST are low after birth and no sex differences in GST content between male and female are observed. At the onset of puberty, however, levels rise to those seen in the adult and the sex differences described above manifest themselves. (Hatayama *et al.*, 1986; Igarashi *et al.*, 1985).

The endogenous regulatory mechanisms which determine GST expression will undoubtedly prove to be complex. To further add to the intricacy of the situation, dietary factors and exogenous compounds can affect the expression of GST. It is becoming increasingly apparent that many chemical compounds with vastly different structures can influence hepatic GST levels. Phenobarbital and 3-methylcholanthrene, classical inducers of the phase I P-450 monooxygenase system have been the most extensively studied xenobiotic inducers with respect to the xenobiotic regulation of GST (for reviews, see Pickett *et al.*, 1987, Pickett and Hu, 1989). Again, the rat has been used as the model system for these investigations, comparatively little is known of their effect on other species.

Insights into the molecular mechanisms by which 3-methlycholanthrene can regulate GST levels within the cell have been obtained by Pickett and his co-workers.[1] The mechanism by which phenobarbital can affect GST expression in the liver is not known. This topic is dealt with in more detail in chapters 5 and 6.

1.10 Aims of thesis

The aims of this thesis were two fold. Firstly, an investigation was undertaken into the endocrine mechanisms underlying the marked sexually dimorphic expression of the YfYf GST in mouse liver. Secondly, a study was undertaken to determine the effect of the xenobiotic phenobarbital, and other phenobarbital-like inducers, on the expression of GST in mouse liver and to determine if the effects of these foreign compounds were mediated via the endocrine system.

1- Pickett (1987), Pickett *et al.* (1987) and Pickett and Lu (1989).

Chapter 2 - Materials and Methods

2.01 Materials

2.01a Chemicals

These were obtained from the following sources;

Amersham International, Amersham,Bucks, U.K.

P^{32} CTP; hexadeoxyribonucleotides

Anderman & Co. Ltd., Kingston-Upon-Thames, Surrey, U.K.

Nitrocellulose.

B.D.H. Chemicals Ltd., Thornliebank, Glasgow, U.K.

Acetic acid; ammonium formate; boric acid; bromophenol blue; chloroform; DEAE-cellulose paper; formaldehyde; glycerol; glycine; HEPES; hydrogen peroxide; methanol; NN' methylenebisacrylamide; NNN N'-tetramethyethylenediamine (TEMED); trichloroacetic acid; orthophosphoric acid; phenobarbital; phenol; polyacrylamide; sarkosyl; sodium acetate; sodium chloride; sodium citrate; sodium dihydrogen orthophosphate; sodium dodecyl sulphate; Tris; Whatman 3mm filter paper; xylene cyanol.

Beechams Research Lab. , Brentford, U.K.

Ampicillin (trade name Penbritin).

Biorad Laboratories, Watford, Herts, U.K.

Tween 20; 4-chloro-1-napthol.

B.R.L., Uxbridge, Middlesex,U.K.

EcoRI; formamide; guanidinium thiocyanate; low melting point agarose; PstI; SalI.

Difco, Detriot, Michigan, U.S.A.

Agar; bactotryptone; yeast extract.

Koch Light Laboratories, Colinbrook, Berks, U.K.

Cumene hydroperoxide.

Kodak Ltd., Kirby, Liverpool, U.K.

Kodak Xar film

Millipore (U.K.) Ltd., Harrow, Middlesex, U.K.

Membranes (0.45μm) for the ultrafiltration of h.p.l.c. buffers.

Sigma Chemical Co., Poole, Dorset U.K.

Bovine serum albumin; 1- chloro-2,4-dinitrobenzene; coomassie brilliant blue R; diethyl pyrocarbonate (depc); dexamethasone; dithiothreitol; ethylenediaminetetra-acetic acid; ethacrynic acid; ethidium bromide; ficoll; gelatin; glutathione; glutathione reductase; glutathione sepharose 6B; isoamyalcohol; lysozyme; magnesium chloride; 2-mercaptoethanol; 3-[N-morpholino]propanesulphonic acid (MOPS); nicotinamide adenine dinucleotide phosphate (reduced form type X); potassium acetate; polyvinyloyrrolidine (PVP); tetracycline: testosterone propionate; polyoxyethylene-sorbitan monolaurate (Tween 20).

2.01b Non commercially obtained material.

Antisera and protein standards

Antisera raised against rat GST subunits and rat GST protein standards for SDS/PAGE and western blotting were kindly provided by Dr. J.D. Hayes from this department. The specificities of the antibodies have been described by Hayes and Mantle (1986a). Antisera towards mouse GST subunits and mouse GST standards for Western Blotting were a kind gift from Dr. L.I. McLellan again of this department. The specificity of the mouse antibodies have also been examined (McLellan and Hayes, 1989).

cDNA Clones

Professor M. Maramatsu, Department of Biochemistry, Tokyo, Dr. C.B.Pickett, Merck Frosst Centre for Therapeutic Research, Quebec and Dr. J.B.Taylor of the CRC Molecular Toxicology Group, Middlesex Hospital, London, generously provided cDNA clones encoding the rat Yf, Yb_1 and Ya subunits respectively. Details of these clones are given in section 2.07.

Chemicals

TCBOP was a gift from Dr. Alan Poland of the McArdle Laboratory for Cancer Research, Maddison, Wisconson, U.S.A.

Human Recombinant Growth Hormone (trade name Genotropin), was a gift from Dr. Richard Wilde of KabiVitrum Ltd.

RNA

Hepatic RNAs isolated from hypophysectomised mice were donated by Dr. C.J. Henderson.

2.01c Animals

A range of experimental animals were used in the course of this work, the sources for mutant mouse strains, surgically prepared animals and their appropriate controls are given below, all other mice were obtained from Banton and Kingman, Grimstone, Hull U.K.

Surgically prepared animals

Hypophysectomised male and female C57BL/6 mice plus sham operated controls were obtained from Charles Rivers Laboratories Willmington, Massachusetts, U.S.A.

Castrated C57BL/6 males plus normal females used in testosterone induction experiments were obtained from Harlon Olac Lmt, Blackthorn, Bicester, U.K.
Castrated and ovarectomised CFLP mice were obtained from Interfauna U.K Ltd., Huntingdon, Cambridgeshire, U.K.

Mutant Mice Strains

"Little Mice" (lit/lit) plus the heterozygote and wild-type controls were obtained from two sources. Preliminary experiments were carried out using mice kindly provided by Dr Rhea Al-Shawi, Dept. of Genetics, University of Edinburgh. Subsequently, mice were commercially obtained from the Charles Rivers Laboratories, Willmington, Massachusetts, U.S.A.
Hypogonadal mice were a kind gift from Professor G. Fink, Brain Metabolism Unit, Department of Pharmacology, University of Edinburgh.
Tfm mutants were obtained from Dr Mary Lyons, M.R.C. Radiobiology Unit, Chilton, Oxon U.K.

Maintenance and Treatment

Adult mice and rats of between 8 and 12 weeks were used in experiments, unless otherwise stated. Animals were housed at either Clare Hall, University of Oxford or at the Faculty of Medicine Animal Area, University of Edinburgh. Once transported, the animals were allowed to acclimatize for at least 2 weeks prior to treatment and were fed (Banton and Kingman Mouse and Rat maintainance diet) and watered ad libitum, the animals were kept in quarantine throughout.

2.02 General Analytical Techniques (Protein)

2.02a Protein determination

The protein concentration of samples was obtained using the method developed by Bradford (1976), with BSA as a calibration standard.

2. 02b GST Enzymatic Assays

Enzyme assays were either performed using a Cobas Fara centrifugal analyser or were determined by manual methods on a Uvikon 860 spectrophotometer.

Glutathione S-transferase activity was determined at 37°C using methods similar to those described by Habig and Jakoby (1981); the assays were normally performed in duplicate or triplicate. Information on conditions under which individual assays for a range of substrates were performed is presented in Table 2.01.

The centrifugal analyser was usually employed to determine the GST activity towards the general substrate CDNB and the GSH peroxidase activities towards either cumene hydroperoxide (CuOOH) or hydrogen peroxide (H_2O_2).

Using this instrument a maximum of 29 samples could be analysed simultaneously for enzyme activity. In the case of GST activity, samples were preincubated with GSH prior to the initiation of the reaction by centrifugation of CDNB into the reaction mixture. Assays were performed in 100mM-sodium phosphate buffer pH6.5 (in a total volume of 250µl), final concentrations of GSH and CDNB were 2mM and 1mM respectively. The conjugation of CDNB with GSH was measured directly by

Substrate	[Substrate] (mM)	[GSH] (mM)	pH	Extinction Coefficient (nM^{-1} CM^{-1})	Wavelength (nm)
1-Chloro-2,4-dinitrobenzene[a]	1.0	2.0	6.5	9.6	340
1,2-Dichloro-4-nitrobenzene[a]	1.0	5.0	7.5	8.5	345
p-Nitropheny acetate[b]	0.2	0.5	7.0	8.8	400
1,2-Epoxy-3-(p-nitrophenoxy)-propane[a]	0.5	5.0	6.5	0.5	360
trans-4-Phenyl-3-butene-2-one[a]	0.05	0.25	6.5	-24.8	290
Ethacrynic acid[a]	0.2	0.25	6.5	5.0	270
Cumene hydroperoxide[c]	1.2	1.0	7.0	-6.2	340

Table 2.01 Conditions for spectrophotometric assays of GST activities. Assays were performed in a thermostated cell compartment at 37°C according to the methods of (a) Habig & Jakoby, 1981; (b) Keen and Jakoby, 1978 and (c) a modification of the method described Wendel (1979), refer to section 2.02b.

monitoring the absorbance at 340nm. A series of 8 absorbance readings were taken, the first 10 sec after mixing, followed by 7 readings at 5 sec intervals thereafter.

Total peroxidase activity in the cell cytosolic fraction (i.e. both the Se-dependent and Se-independent form) was measured using the substrate cumene hydroperoxide (CuOOH). The Se-dependent GSH peroxidase can be specifically assayed using the substrate hydrogen peroxide (H_2O_2) . The difference in peroxidase activity towards these two substrates provides a approximate measure of the Se-independent GSH peroxidase activity contributed by the GST (Lawrence and Burk,1976; Carmagol *et al.*, 1983).

Both assays were carried out on the Cobas Fara centrifugal analyser, using a coupled reaction based on the method described by Wendel(1979). NADP formation, monitored by measuring the absorbance at 340nm is used as an indicator of peroxidase activity by the coupling of 2 reactions, the equations for which are given below:-

$$ROOH + 2GSH \text{ ----------> } H_2O + ROH + GSSG \qquad (1)$$

$$GSSG + NADPH + H^+ \text{ ----------> } 2\ GSH + NADP \qquad (2)$$

Samples were preincubated with initial reagent containing GSH (final concentration- 1mM), NADPH (final concentration- 0.25mM) and GSH reductase (at a final concentration of 1unit/µl) in 60mM-Tris/HCl, 0.12mM-EDTA (pH 7.6). The reaction in a total volume of 340µl, was initiated by the addition of substrate, CuOOH or H_2O_2 to a final concentration of 1.2mM and 0.6mM respectively. Ten absorbance readings at 340nm were taken in both assays. In the H_2O_2 peroxide assay, the absorbance was monitored 5 sec after the initiation of the reaction, with

measurements at 5 sec intervals thereafter. Absorbance in the CuOOH hydroperoxide assay was measured at 10 sec intervals.

Units of enzyme activity for all assays are given as µmoles of product formed /min / mg protein. Values obtained for the non-enzymic conjugation of substrates with GSH were subtracted from initial rates. In the case of assays performed manually, the change in absorbance (ΔA) was calculated per minute and converted to µmoles of product formed using the appropriate extinction co-efficient, details of which are given in Table 2.01.

Linear regression analysis was performed using the manufacturers kinetic rate programme for those assays undertaken on the Cobas Fara. Using a preprogrammed conversion factor, data from the CDNB assay was presented as ΔA/min/ml. As with manually performed assays, the appropriate extinction co-efficient allowed conversion of these units to µmoles of product/min/ml. Peroxidase activity was calculated using a similar programme and presented as µmoles of NADP formed/min/l.

2.02c SDS/Polyacrylamide-Gel Electrophoresis

This was performed essentially as described by Hayes *et al.* (1979), using the discontinuous buffer system developed by Laemmli (1970). Electrophoresis was carried out at room temperature in a Protean II gel electrophoresis system (Bio-Rad Lab Watford, U.K.). Vertical polyacrylamide slab gels (dimensions 0.072cm x 20cm x20cm) were employed which comprised of a resolving gel about 15cm high of 12% (w/v) polyacrylamide containing 0.32% (w/v) NN' methylenebisacrylamide and

0.375M-Tris/HCl buffer pH8.85, over which a 3% (w/v) polyacrylamide stacking gel of 4.5cm containing 0.125M-Tris/HCl buffer pH6.8 was layered; the resolving gel was allowed to polymerise before the stacking was cast. SDS (0.1%) was added to both gel systems.

Cytosols were prepared for electrophoresis by the addition of boiling mix solution containing 3% (w/v) SDS, 30% glycerol, 2% mercaptoethanol and 0.15M-Bromophenol Blue, at a ratio of 2x sample to 1x boiling mix; the final sample preparation mixture contained 1% (w/v) SDS. Samples were heated at 90°C for about 5min and appropriate aliquots loaded onto the gel. Samples were subject to electrophoresis through the stacking gel at a constant 250 volts and then through the resolving gel at 350 volts. Electrophoresis was stopped when the bromophenol dye front reached 0.5cm from the edge of the resolving gel. Proteins were visualised by staining for 30-60min at room temp in a 0.2% (w/v) solution of Coomassie Brilliant Blue R in a water/methanol/acetic acid solution, (50 : 50 : 7 by volume). Gels were then destained by passive diffusion in several changes of a water/methanol/acetic acid solution, (88 : 5 : 7, by volume).

2.02d Western Blotting

Western blotting was carried out using a modified version of the method originally described by Towbin *et al.* (1979).

Polypeptides were resolved by SDS/PAGE as described in section 2.02c. Immediately following electrophoresis the resolving gels were equilibriated for 30min in the transfer buffer (25mM-Tris,193mM-Glycine pH 8.3) to remove SDS. The proteins were electrophoretically transferred from the gel to nitrocellulose paper using a

Bio-Rad Trans-Blot Kit. Transfer was performed at 15°C for 3-4 hours at a constant current of 0.25 amps, using as a power source Bio-Rad model 250/2.5 power pack (Bio-Rad Laboratories). Unbound sites on the nitrocellulose filter were blocked by an overnight incubation in a 3% (w/v) solution of gelatin in TBS buffer (20mM-Tris/HCl, 500mM-NaCl pH 7.9).

After washing with TBS to remove gelatin, the nitrocellulose filters were incubated at room temperature with primary antibody for 1-3 hours. The time of incubation and antibody titre were dependent on the antisera used; antibodies were diluted in TBS with 1% (w/v) gelatin and 0.05% (v/v) Tween 20. Filters were washed (4 x 15min) with TBS to remove unbound first antibody. Primary Antibody - Antigen complexes were detected with a double antibody EIA system using goat anti-rabbit IgG horseraddish peroxidse, obtained from Bio-Rad Laboratories, with 4-chloro-1-napthol as the substrate, according to the manufacturers instructions.

2.03 Statistics

The degree of statistical significance of results was assessed using Student's t-test which was performed using the Statworks programme on the Apple MacIntosh microcomputer.

2.04 Cytosol Preparation

Tissue was finely minced by hand, followed by homogenisation in ice-cold 20mM-Tris/HCl pH7.8. In the case of pooled samples, tissue was homogenised at a ratio of 1 part tissue to 3 parts buffer. By contrast, livers which were analysed individually were homogenised in 10 vol homogenisation buffer. Homogenates were centrifuged at

100,000g for 1 hour. The supernatants obtained after centrifugation were retained, these are referred to as the cytosolic fraction of the cell. Cytosols were then aliquoted and stored at -70°C unless destined for GSH affinity chromatography. If purification of the GST fraction was being undertaken the cytosols were dialysed against 50mM-Tris/HCl, 200mM-sodium chloride, pH 7.8 containing 0.5mM-DTT over a 16 hour period.

2.05 GST Purification.

The method used was based on the purification procedure described by Hayes *et al.* (1979) using glutathione affinity chromatography followed by hydroxyapatite h.p.l.c. to resolve individual mouse GST subunits.

2.05a Glutathione Affinity Chromatography

A one-step purification of mouse hepatic GST from the cytosolic fraction of the cell was undertaken at 4°C using glutathione affinity chromatography (Simons and Vander Jagt, 1981). The dialysed cytosol was applied to a 1.6cm x 20cm glutathione Sepharose 6B column, which had previously been equilibriated at 4°C in running buffer, 50mM-Tris/HCl, 200mM-NaCl pH7.8. After application the non-specifically absorbed material was removed by washing the column with 2-3 column volumes of column running buffer. Protein that was specifically retained by the affinity matrix was eluted with one column volume of 250mM-Tris/HCl buffer pH8.8 containing 40mM GSH. The GST-containing fractions were identified by measuring CDNB activity. The protein content of the eluate was also determined by the method of Bradford (1976). Fractions containing high CDNB conjugating activity were combined and prepared for hydroxyapatite chromatography by dialysis at 4°C

against two changes of 2.5 litres of 10mM-sodium phosphate buffer pH6.7 containing 0.5mM-DTT for 16 hours.

2.05b Hydroxyapatite HPLC

Individual isoenzymes were resolved from the GST affinity purified pool purified using hydroxyapatite h.p.l.c. After dialysis, the pooled GST fractions were injected onto a Bio-Gel HPHT (h.p.l.c. grade) column (obtained from Bio-Rad Laboratories, Watford, Herts, U.K). This column was eluted at 0.5ml/min and developed using a 10 - 350mM gradient of sodium phosphate buffer pH6.7; both the running buffer and the limit buffer contained 0.5mM-DTT and 0.1mM-calcium chloride throughout. Peaks were detected by monitoring the eluate at 280nm. Fractions of 0.5ml were collected and their CDNB-GSH conjugating activity and protein concentrations were measured.

2.06 Measurement of Plasma Hormone Levels

2.06a Measurement Of Plasma Testosterone Levels

Radioimmunoassay of plasma ether extracts using a specific anti-testosterone antiserum and I^{125} testosterone-3-carboxymethy-oxine radiolabel was used to measure circulating testosterone levels in little mice and control samples according to the method of Corker *et al* .(1978).

These assays were very kindly performed by Dr. Sadie Gow from the Radioimmunoassay section, Department of Clinical Chemistry, University of Edinburgh.

2.06b Thyroid Hormones

Plasma T_3 levels were measured using a double antibody RIA as described by Ratcliffe *et al.* (1974).

T_4 measurements were carried out by Dr. C.J. Henderson from the ICRF Molecular and Pharmacolgy Laboratory again by the method of Ratcliffe *et al.*, (1974).

2.07 cDNA Clones

Hepatic GST mRNA levels in mouse and rat were investigated by Northern Blot analysis using as probes cloned cDNAs from each of the three rat cytosolic gene families. Clones containing complete amino acid sequences for the rat Yb_1 and Yf subunits were used to detect mu and pi class liver mRNAs respectively. The alpha class mRNAs were measured using pGSTr155 which contained a partial cDNA insert coding for the N-terminal 129 amino acids of the rat Ya subunit. More detailed information on these clones is given in table 2.02. Figure 2.01 contains the restriction enzyme maps for GST cDNA inserts cloned.

2.08 Maintenance And Culturing Of Plasmids

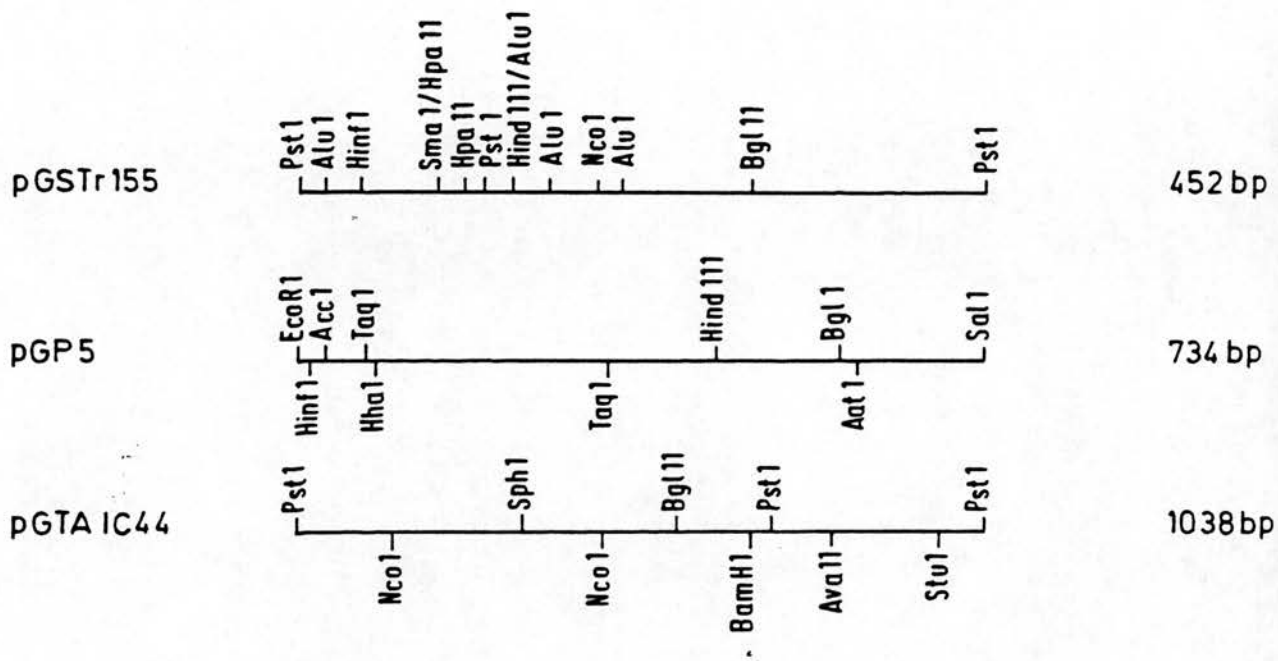
2.08a Selection and short term culture

For stable maintenance and culturing, plasmids were transformed using the calcium chloride method (Mandel and Higa, 1970) into the Eschrichia Coli K12 strain HB101. Successfully transformed cells were selected and maintained on L-agar plates in the presence of the appropriate antibiotic. Tetracycline (at a concentration of 10μg/ml)

PLASMID	VECTOR	ANTIBIOTIC RESISTANCE	GST cDNA CLONED	INSERT SIZE	CLONING SITE(S)	REFERENCE
pGSTr155	pAT153	TETRACYCLINE	partial cDNA encoding N-terminal 153 αα of rat Ya subunit	452bp	Pst1	Taylor *et al.* (1984)
pGP5	pUC8	AMPICILLIN	complete αα seq for the rat Yf subunit	734bp	EcoR1/Sal1	Sugoaka *et al.* (1985)
pGTA/C44	pBR322	TETRACYCLINE	complete αα seq for the rat Yb_1 subunit	1038bp	Pst1	Ding *et al.* (1985a)

Table 2.02 A description of the rat Glutathione S-Transferase Clones used for Northern Blotting.

Figure 2.01. Restriction enzyme maps of glutathione S-transferase cDNA clones pGSTr155, pGP5 and pGTA/C44



was used to select for transformants containing the plasmids pGTA/C44 and pGSTr155, whereas ampicillin (100μg/ml) was used to select for pGP5. Running cultures were stored at 4°C and single colonies were subcultured every 4 weeks by overnight growth at 37°C.

2.08b Long Term Storage Of Plasmids

Plasmid-transformed cells were grown overnight at 37°C using the appropriate selection marker. Sterile glycerol to a concentration of 30% (v/v) was added to the culture and the resulting solution was divided into aliquots and frozen at -70°C for storage. Plasmid DNA was also prepared as described in Section 2.09 and stored at -70°C.

2.08c Bacterial Media

L broth and L agar (Lennox ,1955) were used for liquid and solid phase culture of HB101 respectively.

L broth (and agar)	
Difco bactotryptone	10g l^{-1}
Difco yeast extract	5g l^{-1}
NaCl	5g l^{-1}
(Difco agar	15g l^{-1})

Plasmid-containing HB101 were always cultured in the presence of the selectable antibiotic.

2.09 Preparation Of Nucleic Acids

2.09a Large Scale Preparation Of Plasmid DNA

The procedure used was a modified version of the small scale DNA plasmid preparation developed by Birmboin and Doly (1979).

A single colony was inoculated into 10ml of L broth which contained the relevant antibiotic and incubated at 37°C for 6-8 hours. This culture was then added to a flask containing a further 500ml of L broth and antibiotic. The inoculum was left overnight at 37°C shaking vigorously. Cells were harvested by centrifugation at 8,000g (4°C) for 5min. The cellular pellet was resuspended in 18ml of Solution A (see below for recipe) and incubated at room temperature for 10min to lyse the bacterial cell wall. Solution B (40ml) was added and the mixture left on ice for 5min after which Solution C (20ml) was added and the preparation left on ice for a further 15min. Treatment with these solutions disrupted the bacterial cell membrane and precipitated the proteins. The heavy precipitate formed was pelleted by centrifugation at 8,000g (4°C) for 5min. The plasmid containing supernatant was retained. To precipitate the DNA, cold isopropanol (0.6 vols) was added to the supernatant and the solution left at room temperature for 15min. Nucleic acids were pelleted by a 5 minute centrifugation at 8,000g. The resulting pellet was resuspended in 6.7ml of TE. To this resuspended solution 7.4g of ceasium chloride and 1.4ml of a solution of ethidium bromide (10mg/ml) were added. The DNA was banded by ultracentrifugation at 200,000g for 16hr at 16°C and after centrifugation the resolved nucleic acid bands were visualised using a short wavelength transilluminator. The band consisting of closed circular plasmid DNA was collected using a hypodermic needle inserted into the side of the ultracentrifuge tube as

described in Maniatis *et al.* (1982). Ethidium bromide was removed by extraction with isoamylalcohol and the ceasium chloride removed by dialysis against 3 changes of 1000 x vol TE at 4°C for 16hr. Plasmid DNA was stored at -70°C.

Solution A		Solution B	
Lysozyme	5mg ml-1	SDS	1%
Glucose	50mM	NaOH	0.2M
Tris HCl pH 8.0	25mM		
EDTA	10mM		

Solution C		TE	
KOAc (pH4.8)	3M	Tris/HCl (pH8.0)	10mM
Acetic Acid	2M	EDTA	0.1mM

2.09b RNA Preparation

The extraction of hepatic RNA was undertaken using a modified version of the guanidinium thiocyanate method described by described by Berger (1987).

In order to avoid RNA degradation by contaminating nucleases, every item of plastic or glassware brought into contact with the RNA was steeped in water containing 0.1% depc for 1-2hr and then autoclaved. For reasonable yields of undegraded RNA, it was also necessary to snap freeze liver tissue in liquid N_2 immediately after sacrifice.

Volumes of reagents quoted below are appropriate for the processing of 0.5g of liver. When more tissue was available, the preparation was scaled up accordingly.

Liver tissue (0.5g) was minced finely and homogenised, using a teflon glass homogeniser, in 1ml of Solution D. To this homogenate the following solutions were added sequentially :-

0.1ml 2M-sodium acetate (pH4.0)

1.0ml phenol (saturated with depc treated water)

0.2ml chloroform / isoamylalcohol (49:1)

This suspension was mixed thoroughly and cooled on ice for 15min. Samples were centrifuged at 10,000g for 20min at 4°C. The aqueous phase was transferred to a clean tube and the RNA precipitated by the addition of an equal volume of cold isopropanol, this solution was placed at -20°C for 1hr. To pellet the RNA, the precipitate was centrifuged as described above. The pellet was redissolved in 0.3ml of Solution D and reprecipitated with isopropanol under the conditions used previously. An eppendorf microfuge was used to pellet the RNA, spinning for 4 min at 4°C. The pellet was washed twice in 75% ethanol and dried under vacuum. Finally, the RNA was dissolved in depc treated H_2O and stored at -70°C. Measurement of RNA concentration was performed as described in Section 2.10.

Solution D

4M-Guanidinium Thiocyanate

25mM-Sodium Citrate (pH7.0)

0.5% Sarcosyl (v/v)

100mM-mercaptoethanol.

2.10 Quantitation of DNA and RNA

2.10a Spectrophotometric Determination

DNA and RNA concentrations were measured spectrophotometrically at 260 and 280nm. Concentrations of nucleic acid given below correspond to a change of 1 absorbance unit.

1 absorbance unit at 260nm = 50μg/ml for double stranded DNA

= 40μg/ml for single stranded DNA and RNA

The ratio between absorbance at 260 and 280nm provides an estimate of the purity of the preparation. For a pure DNA preparation, OD_{260}/OD_{280} should be 1.8, for RNA, the ratio should be 2.

2.10b Ethidium Bromide Fluorescent Quantitation Of Double Stranded DNA

DNA concentration was also estimated by running samples on a 0.8% (w/v) midi-agarose gel using BRL horizontal electrophoresis kit H4 (BRL, Uxbridge U.K.). Preparation of gels and the conditions under which they were run are as described for mini-agarose gel electrophoresis in Section 2.11. The amount of U.V. induced-fluorescence emitted by ethidium bromide molecules intercalated with DNA is proportional to the total mass of DNA. The quantity of DNA in the sample can be estimated, therefore, by comparing the fluorescent yield of the sample with that of a series of standards.

2.11 Mini-Agarose Gel Electrophoresis

For the rapid analysis of restriction digests and the isolation of cDNA inserts (section 2.12b), mini-agarose gels were run on a Bio-Rad gel kit, model H6. Gels containing 0.8% (w/v) agarose in 1xTBE with 0.5μg/ml (dimensions 50mm x75mm x 5mm) were cast on a glass tray, transferred to the gel kit and run in 1XTBE buffer at 20v for 2-3hrs. DNA fragments were visualised using a short wavelength transilluminator (Model TM-40 from Ultravoilet Products Incorporated).

TBE

130mM -Tris

45mM -Boric Acid

2.5mM -EDTA

2.12 Northern Blotting

2.12a Electrophoretic separation, transfer and hybridisation of RNA

To measure hepatic GST mRNA levels, northern blot analysis was performed using as probes the rat cDNAs described in section 2.07 (also table 2.02).

RNA was electrophoretically size-fractionated under denaturing conditions using the system developed by Lehrach *et al.* (1977). Horizontal 1% (w/v) agarose gels in 1x gel running buffer with 2.2M-formaldehyde were prepared (dimensions 20cm x 25.0cm x 0.5cm). Prior to electrophoresis, RNA samples in gel running buffer

with 2M-formaldehyde, 50% (v/v) formamide were incubated at 55°C for 15min. Loading dye (2ml) was added to samples directly after heating. A midi-formaldehyde gel was prepared to check the quality of the RNA and sample loadings prior to Northern Blot analysis. After electrophoresis the gels were stained in ethidium bromide for 1-2hr and the ribosomal bands were visualised using a short wavelength transilluminator.

Electrophoresis was performed, using BRL horizontal electrophoresis model H1 (BRL, Uxbridge, U.K.) at 45v for approximately 16hr, until the dye front was 5cm from the edge of the gel. RNA transfer from formaldehyde gel to nitrocellulose occurred by capillary action in 10xSSC as described by Southern (1975), after which the filter was baked at 80°C for 2hr in a vacuum-oven to fix the RNA.

To check that transfer had occured successfully, the gel was stained in ethidium bromide (0.5µg/ml) for 2-3 hr, washed in water and viewed using a short wavelength transilluminator. When RNA is size fractionated under denaturing conditions and stained in ethidium bromide, two bands corresponding to 18s and 5s ribosomal RNA are clearly visible. Absence of these bands after transfer was indicative that the process had been successful.

After fixing, the nitrocellulose filter was prehybridised at 65°C for 4 -6 hr in a heat-sealable plastic bag. The prehybridisation solution used was as below:-

Prehybridisation Mix

50x Denhardts solution	2ml
20x SSPE	7.5ml
10% SDS	0.25ml
dH2O	14.9ml
single stranded herring sperm DNA (10mgs/ml)	0.25ml

Probes, prepared and radioactively labelled as described in sections 2.12b and 2.12c, were added directly to the prehybridisation solution and allowed to hybridise for 16-24hr at 65°C in a shaking water bath. Unbound probe was removed by washing twice in 2xSSPE / 0.1%SDS for 30min at 65°C. The nitrocellulose filters were air-dried, wrapped in Saranwrap and autoradiographed using Kodak Xar5 film. Filters were exposed between a few hours and 2 wk depending on the probe and the samples analysed.

5 X Gel Running Buffer

0.2M-MOPS (pH7.0)

50mM -Sodium acetate

5mM-EDTA

Loading Dye

0.4% bromophenol blue

0.4% xylene cyanol

50% glycerol

1mM-EDTA

20 X SSC

sodium chloride 3M

trisodium citrate 0.3M

20 x SSPE

sodium chloride 3M

sodium dihydrogen orthophosphate 0.18M

50 x Denhardts Solution

Ficoll 10g l^{-1}

PVP 10g l^{-1}

BSA 10g l^{-1}

2.12b Preparation Of cDNA Probes

cDNA probes for northern blot analysis were radioactively labelled by the 'Oligo-labelling' technique developed by Feinberg and Vogelstein (1983). DNA probes were denatured and labelled with the large fragment of DNA Polymerase 1 using random

oligonucleotides as primers for the DNA synthesis reaction. In the case of pGSTr155, total plasmid was used as a probe for alpha class mRNA levels. cDNA inserts were isolated for the plasmids pGP5 and pGTA/C44, labelled and used to probe pi and mu class mRNAs respectively.

When total plamid is required in the labelling reaction, it is necessary to linearise the closed circular DNA molecules by restriction with a nuclease which cuts at a unique site.

pGSTr155 (1.4mgs) was digested with 2 units of the restriction enzyme EcoR1 in a buffer containing 50mM Tris-HCl pH8.0, 10mM MgCl2, 100mM NaCl at 37°C for 5 -7hr. The reaction was stopped by heating at 70°C for 10min. This reaction mixture was stored at -20°C, aliquots being taken and added to the labelling reaction when required.

To check that digestion had gone to completion, 50ng of digested DNA, together with an uncut plasmid control was subjected to electrophoresis on a 0.8% mini-agarose gel, as described in section 2.11. Unrestricted, closed and circular plasmid DNA segregates into three distinct bands during electrophoresis. These correspond to, in order of their mobility on an agarose gel, a) the supercoiled form b) the linearised form of the plasmid and c) partially nicked plasmid. Successfully restricted DNA runs as one distinct band on electrophoresis, which migrates at the same mobility as the linearised form.

Plasmids pGP5 and pGTA/C44 were restricted with the appropriate nucleases to release the cloned cDNA inserts.

Plasmid pGP5 (1.4mgs) was digested for at 37°C for 5-7hr with 2 units of the restriction endonucleases, SalI and EcoRI in 50mM-Tris/HCl buffer pH8.0 containing10mM-$MgCl_2$, 100mM-NaCl for 5-7 hr. Digestion released the complete insert of 725bp

Plasmid pGTA/C44 (1.4mgs) was digested under the same conditions. The restriction enzyme, PstI was used to release the insert. As this enzyme works better in a low salt environment, NaCl to a concentration of 50mM, rather than 100mM, was added to the restriction buffer used for the SalI and EcoRI.

Due to an internal PstI site in the Yb_1 cDNA insert, the insert is cleaved into 2 fragments of 747 bp and 291bp. The high molecular weight fragment was used as a probe for northern blot analysis. A detailed restriction map of the cDNA inserts for the clones used is given in Figure 2.01.

Loading dye (the recipe is given in section 2.012a) was added to the digests and samples were subject to electrophoresis in a mini-gel containing 0.8% (w/v) low melting point agarose and 0.5 µg/ml ethidium bromide using conditions described in Section 2.11. Segments of the gel containing the desired DNA fragments were excised and heated to 100°C for 10 min to melt the agarose and denature the DNA. Isolated fragments of DNA in low melting point agarose were stored at -20°C and reboiled for 3 min prior to an aliquot being added to the labelling reaction.

2.12c Radiolabelling Of cDNA Probes

The 'oligo-labelling' technique described by Feinberg and Vogelstein (1983) allows the labelling of small amounts of DNA to a high specific activity. Routinely 90ng of

the purified cDNA inserts contained within low melting point agarose were used in the labelling reaction. For pGSTr155, where both the vector and insert were labelled, 150ng of linearised plasmid was added to the reaction. Labelling was performed at room temperature in a total volume of 30µl, the constituents of the labelling reaction and necessary solutions are as follows:-

Radiolabelling Reaction Mixture

OLB	3ml
BSA (1mg-1)	2ml
Klenow enzyme	1unit
P^{32}dCTP	50µCi

90ng of cDNA insert or 150ng total plasmid, made up to a final volume of 30µl with water.

The radiolabelling reaction was allowed to proceed for approximately 9hr. The DNA was then denatured by boiling the solution at 100°C for 5min. Probes were now ready for hybridisation and were directly added to the prehybridising filter as described in section 2.12a.

The amount of radioactivity incorporated into DNA was estimated by DEAE-cellulose paper chromatography in 0.3M-ammoniun formate pH8.0. An aliquot (1µl) of the labelling reaction was spotted onto DEAE-cellulose paper and allowed to dry. The chromatograph was run for approximately 30min, air dried, and autoradiographed for 20min at room temp, using Kodak Xar5 film. Labelled probe remained at the origin, while unincorporated nucleotides ran close to the solvent front. Incorporation of radioactive nucleotides routinely ranged from between 50 - 70%.

OLB

OLB is a complex buffer produced from Solutions A, B, C, which are mixed in a ratio of 2 : 5 : 1. This stock solution can be stored at -20°C for up to 3 months.

Solution A. 625ml 2M-Tris/HCl (pH 8.0) + 25ml 5M-MgCl2 + 350ml dH_2O + 18ml mercaptoethanol + 5ml of dATP, dGTP, dTTP.

(Each triphosphate is dissolved in 3mM-Tris/HCl, 0.2mM EDTA (pH7,0) at a concentration of 0.1M.)

Solution B 2M-HEPES titrated to pH6.6 with NaOH. This solution is stored at -4°C.

Solution C Hexadeoxyribonucleotides evenly suspended in 3mM-Tris/HCl, 0.2mM- EDTA (pH7.0) at a concentration giving 90 OD units/ml and stored at -20°C.

Chapter 3 Regulation of the Hepatic Yf GST Subunit by Androgens

3.01 Introduction

3.01a Sexual Differentiation Of Hepatic Proteins

Over the last few decades, it has become increasingly evident that the liver is a sexually differentiated organ. Numerous examples now exist of a wide range of liver proteins, involved in many diverse functions, that are expressed at different levels in males and females. For an excellent review, see Roy and Chatterjee (1983). Table 3.01 contains a list of some of the best documented examples of sexual dimorphism in both rat and mouse. In some cases this difference can be very slight, for example, the developmentally regulated cytochrome P-450 f is present at higher levels (approximately two-fold) in female rat liver microsomes than in male liver microsomes (Gonzalez *et al.*, 1986). Alternatively, it can be very marked and α_{2u} globulin presents one of the most dramatic examples of hepatic sexual differentiation in the rat. This globulin is the major protein species in male urine, constituting approximately 50% of total protein and is essentially absent from the urine of female rats; it has been estimated that male rats secrete over 20mg of α_{2u} globulin per day. Synthesis occurs in the liver where the mRNA species encoding α_{2u} globulin has been estimated to form 1-2% of total liver mRNA. In the female, the amounts of this protein synthesized are barely detectable (Roy and Neuhaus, 1966, 1967; Chattergee and Roy, 1980; Roy and Chattergee, 1983).Examples of sexual dimorphism in non-gonadal tissues are not simply confined to the liver. Other tissues, particularly the kidney and brain also display a marked degree of sexual

Table 3.01 Examples of liver functions showing at least a fivefold sex difference

Liver Parameter	Male	Female
Receptors		
Prolactin		+
Secretory proteins		
Urinary α_2-microglobulin	+	
Mouse major urinary protein	+	
Enzymes		
Δ4-3-oxostroid 5α-reductase		+
Carbonic anhydrase III	+	
P-450 16α	+	
P-450 15β		+
P-450 g	+	
P-450 pcn 2	+	
P-450 a 1		+
P-450 a 2	+	
Mouse C-P-450 16α	+	
Mouse I-P-450 16α		+
Mouse P-45015α		+

taken from Zaphiropolous *et al.* (1989).

differentiation at many levels. The androgenic regulation of various tissues has been reviewed by Bardin and Caterall (1981).

The liver is also the body's major centre for the detoxification of xenobiotic compounds. Many of the enzymes expressed in the liver are involved in the biotransformation of drugs, carcinogens and other environmental pollutants. It has long been noted that the duration of effect of a variety of drugs was dependent on the sex of the animal used in the experiment. As long ago as 1932, Nicholas and Barron (1932) reported a sex difference in the metabolism of the barbituate amobarbital. These workers showed that female rats required only half the dose of barbituate to induce anaesthesia as compared to male rats. In a similar vein, Holck *et al.* (1937) noted that the sleeping time for a standard dose of barbituate was longer in females than in males. This difference in the effect of drug dosage and duration of action was later shown to be due to faster metabolic clearance of these compounds by the liver in male rats (Crevier *et al.*,1950 ; Pellerin *et al.*,1954). Many reports now exist in the literature of other drugs which display a similar dimorphism, a list is given in Table 3.02

Differences in the metabolism of many compounds result from the sex-specific expression of individual members of the cytochrome P-450 monooxygenase system. These enzymes are also responsible for the very marked sex differences also observed in steroid metabolism, since steroids serve as the natural substrates for many of the cytochrome P-450s. (For reviews concerning sex differences in drug and steroid metabolism and the regulatory mechanisms behind these differences see Skett and Gustafsson, 1979; Gustafsson *et al.*,1983; Skett, 1987) Although the rat has been the most fully characterised species with respect to sexual dimorphism and the underlying mechanism whereby these differences are generated,

Table 3.02 Sex differences in xenobiotic metabolism in the rat

Substrate	Action
Aflatoxin b_1	potent carcinogen
2-Aminophenol	aniline metabolite
Aniline	solvent
Dextrorphan	cough supressant
Diazempan	sedative
Digoxin	cardiac intropic agent
Dinitrotoluene	carcinogen
Ethylmorphine	analgesic
Hexachlorobenzene	fungicide
Imipramine	anti-depressant
Lignocaine	local anesthetic
1-Naphthol	antiparacitical
4-Nitrophenol	fungicide
Phencyclidine	potent analgesic
Phenytoin	antiepilectic agent
Pregnenolone	progestin
7-Propoxycoumarin	flavour enhancer
Testosterone	androgen
Tiaramide	analgesic

taken from Skett (1987).

the sex-specific expression of drug and steroid enzymes has also been described in man (Pfaffenberg and Horning, 1977; MacLeod *et al.*, 1979) and in mouse (Noordhoek, 1972; Brown and Greene, 1980; MacLeod and Shapiro,1989). The mouse, for reasons which will be detailed later, is becoming a very valuable tool for endocrine research and the evidence presented to date suggests it is very similar to the rat in respect to the control hepatic sexual differentiation (Norstedt and Palmiter, 1984; MacCleod and Shapiro, 1989).

Phase II Drug metabolising enzymes have also been reported subject to sexual dimorphism. Differences in the glutathione S-transferases have been dealt with in section 1.09 and will be referred to below. Other phase II drug metabolising enzymes have also been shown to exhibit sexual dimorphism. The glucuronyltransferases were shown to have higher activity towards certain substrates in males compared to females (Inscoe and Axelrod; 1960; Graham and Skett,1987). The sulphotransferases are also subject to sexual differentiation (Skett, 1987).

Most cytochrome P-450 catalysed reactions are performed more efficiently in male than female rat liver. In the rat, two specific cytochrome P-450s are subject to a marked sexual differentiation and these have proved to be very valuable in regulatory studies into the mechanism behind this phenomenon. Cytochrome P-450 16α, which is active in the 16α hydroxylation of testosterone, is regarded as a male-specific marker, with a level of expression up to 20-fold higher in males than is observed in the female (Morgan *et al.*, 1985[a,b]). Cytochrome P-450 15β, which is active on steroid sulphates is virtually absent in the male but accounts for up to 40% of total P-450 in the female liver (Gustafsson and Ingelman-Sundberg, 1974; MacGeoch *et al.*, 1984). These P-450 markers will be used on occasion to illustrate general points in the regulation of sex differences in the liver. In the

mouse, sex-specific expression of the cytochrome P-540s has also been reported but interestingly it occurs in the opposite orientation. Hepatic oxidative metabolism of xenobiotics is greater in female mice than in male mice(MacCleod and Shapiro, 1989).

3.01b Imprinting Of Hepatic Sex Differences

Differences in drug and steroid metabolism are expressed in a developmental fashion. In the pre-pubertal period, no significant differences in drug metabolism are noted between male and female rodents. At the onset of puberty however, the adult pattern of expression becomes manifest in both sexes. This seems to be the general pattern for sexually differentiated hepatic proteins. Again α_{2u} globulin serves as a particularly striking example. This protein is found in trace amounts in the pre-pubertal male, less than 4ng/mg of hepatic protein. At the onset of puberty, expression of α_{2u} globulin rapidly rises resulting in 1000-fold higher levels of the protein in mature rat livers (Roy, 1973; Chattergee *et al.*, 1979)

Yates *et al.* (1958) suggested that androgens were involved in the regulation of sex differences. They reported that Δ^4-3 oxosteroid 5α-reductase activity, which is more highly expressed in female rat liver, was increased towards female levels in male rats following castration. Replacement therapy with the major male sex hormone, testosterone could reverse this effect. Since this work, the ability of testosterone to overcome the effects of castration has been reported for a number of sexually differentiated functions. In most cases, castration of the adult male did not alter enzyme activities completely to the female level but resulted in an activity which lay somewhere between that of males and females. Administration of testosterone could induce activities back to normal male levels. However, when the

castration was performed in the neonatal period, a completely feminised pattern of drug metabolism was observed in adulthood, which could not be altered by testosterone replacement either in the pre-pubertal period or in adulthood. If, on the other hand, the neonatal castrates were administered testosterone directly after testectomy, enzyme activities were restored to that observed in adult castrated rats. Further treatment with testosterone completely restored the normal masculine phenotype. This sequence of events has now been well documented for a number of drug and steroid activities, see reviews by Skett and Gustafsson, (1979) and Gustafsson *et al* (1983).

Thus, the neonatal period is a crucial time in the development of a sexually differentiated phenotype and access to androgens is critical for the establishment of a male-specific pattern of drug and steroid metabolism. This early exposure in the male to androgens has two roles. Firstly, it "imprints" the male pattern of hepatic protein expression which develops after puberty, if this programming is prevented then a feminisation of liver metabolism is observed. Secondly, the ability of the adult to respond to androgen in the adult period is also determined at this time (Einarson *et al.*, 1973; Gustafsson and Stenberg, 1974 a,b).

To summarise, androgens as regulatory factors in drug metabolism are involved at two stages. Firstly, they are the imprinting agents which set the male pattern of expression in the neonatal period. Secondly, they are inducers and regulators of enzyme levels in the adult period.

3.01c Regulation Of The Yf GST Subunit In Mouse

Sex differences in hepatic GST content have been reported in both the rat (Kaplowitz 1975; Hales and Neims, 1976;Lamartiniere1981) and the mouse (Hatayama *et al.*, 1986; McLellan and Hayes, 1987). The mouse displays the most pronounced sex-specific difference in GST content, with the Yf subunit expressed in male liver at levels approximately 10-fold higher than the female. In the male, YfYf constitutes around 70% of total hepatic GST content, whereas in the female it represents only a minor form. This GST subunit has aroused a great deal of interest because of its possible role as a tumour marker. In normal rat liver, both male and female, this subunit is present at essentially undetectable levels, but is found in high concentrations in hepatic preneoplastic nodules (Kitihara *et al.*,1984; Satoh *et al.*, 1985).

The main objective of the work described in this chapter and the next was to investigate the mechanism which determines the sexual differentiation in the levels of the Yf subunit in male and female mouse liver. In view of previous findings with other drug-metabolising enzymes which have been detailed above, a study was undertaken to investigate the possible androgenic regulation of the Yf subunit. Specifically, experiments were performed to determine if the Yf subunit is regulated by the major male sex hormone testosterone and whether this process is subject to imprinting. The role of the pituitary in this process was also examined, this subject will be introduced and the results presented in chapter 4.

3.01d Strategy

The endocrine system is a complex network consisting of numerous hormones which are very tightly controlled as the effects they regulate are fundamental to the proper development and maintenance of the organism and indeed in some cases to its survival. Two approaches were used to 'dissect' this system and allow the study of a limited part relating to the the sex steroids.

1. The GST profile of surgically prepared animals, undergoing castration or ovarectomy was examined and the effect of testosterone replacement therapy was investigated. Two different strains of mice were investigated. CFLP mice were chosen as these mice are somewhat larger than the normal inbred strains (approximately 1.5 times larger than normal C57BL/6 mice). A group of these mice were castrated at 2 weeks of age; the extra size aided surgery. C57BL/6 mice were also examined. This mouse strain is the progenitor strain for the little mouse mutant described in chapter 4 and has been thoroughly characterised with respect to hepatic GST content.

2. Another line of approach available to the endocrinologist, which is proving an increasingly powerful tool, is the use of mouse strains defective in specific endocrine pathways. An excellent account of the endocrine-defective mouse strains that are available to the experimenter is given by Charlton (1984). The hepatic GST content of two strains of mice which either did not produce androgen or were resistant to its effects were examined. Fuller descriptions of the underlying genetic defects which afflict these mice are given in the appropriate results section (see section 3.04a). However, to summarise the defects briefly, the hypogonadal mouse (hpg) is deficient in the production of the pituitary hormones LH and FSH (Luteinizing Hormone and Follicle Stimulating Hormone). These hormones are

essential for the stimulation of the production of testosterone from the Leydig cells of the testes. Thus, these mice do not produce androgen in effective quantities. The testicular feminised mouse (tfm) is deficient in the androgen receptor, which mediates many of the varied actions of testosterone. Therefore in tfm mice these androgen-receptor mediated process do not occur.

3.02 Androgen Regulation of CFLP Mice

3.02a Treatment of animals

Male CFLP mice were sham operated or castrated at 2 or 8 weeks of age. At the age of 11 weeks all animals were either subjected to treatment with testosterone propionate or with the vehicle, propylene glycol. Animals were injected subcutaneously with an initial dose of 2mg/kg testosterone propionate which was followed by 4 daily injections of 1mg/kg. Following a break of 2 days during which testosterone was not administered, 2 further daily injections of 1mg/kg testosterone propionate were given. Twenty-four hours after the final injection, animals were sacrificed by cervical dislocation and liver, kidneys and testes removed quickly frozen on dry-ice and stored at -70°C.

Female CFLP mice were sham operated or ovarectomised post-pubertally. At 11 weeks of age, females were administered either testosterone propionate or vehicle as has been described above for male CFLP mice. Table 3.03 contains a summary of the treatment groups which resulted from these divisions and the number of animals per group.

Table 3.03 : A summary of the experimental animal treatments described in Section 3.02a for CFLP mice

Group	Treatment	No. animals/group
Male sham operated 2 weeks	propylene glycol	4
Male sham operated 2 weeks	testosterone propionate	3
Male castrated 2 weeks	propylene glycol	4
Male castrated 2 weeks	testosterone propionate	4
Male sham operated 8 weeks	propylene glycol	4
Male sham operated 8 weeks	testosterone propionate	4
Male castrated 8 weeks	propylene glycol	2
Male castrated 8 weeks	testosterone propionate	4
Female sham operated	propylene glycol	4
Female sham operated	testosterone propionate	4
Female ovarectomised	propylene glycol	4
Female ovarectomised	testosterone propionate	3

To aid clarity, results will be presented as 3 sections;

1. Male CFLP mice surgically operated upon at 2 weeks of age.
2. Male CFLP mice surgically operated upon 8 at weeks of age.
3. Female CFLP mice.

Section 3.02b Analysis of the Hepatic GST Complement from CFLP Mice

Hepatic cytosols were prepared (see section 2.04) from CFLP mice treated as described in section 3.02a. All livers were processed on an individual basis. Analysis of hepatic GST was carried out using enzyme activities, SDS/PAGE and western blotting with antisera raised against the mouse Yf subunit.

Specific activities towards CDNB were determined for cytosols prepared from individual CFLP mice, the results are presented in figure 3.01. Activity in control sham operated males in both the 2 and 8 week groups were approximately 2.5-fold higher than in control females. Castration at both 2 and 8 weeks led to a 2-fold decline in CDNB activity in the 11 week old adult male. Treatment with testosterone did not markedly reverse this effect. Ovarectomy, testosterone administration or both treatments combined did not affect the relatively low levels of CDNB in the female.

Hepatic cytosolic proteins were analysed by SDS/PAGE (figure 3.02). This revealed the presence a polypeptide Mr 24,800 which co-migrated during electrophoresis with the subunit from purified mouse GST YfYf. This subunit was highly expressed in control male mouse livers. SDS/PAGE showed that castrated males, both at 2 weeks and 8 weeks, possessed markedly reduced levels of this protein in comparison to control males (figures 3.02a, 3.02b). Testosterone treatment did not significantly

Figure 3.01

Levels of CDNB activity in gonadectomised CFLP mice in combination with testosterone replacement therapy.

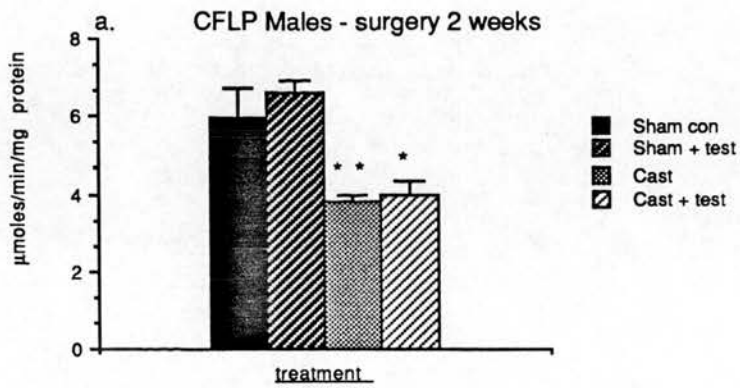
Mean values for each group are quoted.

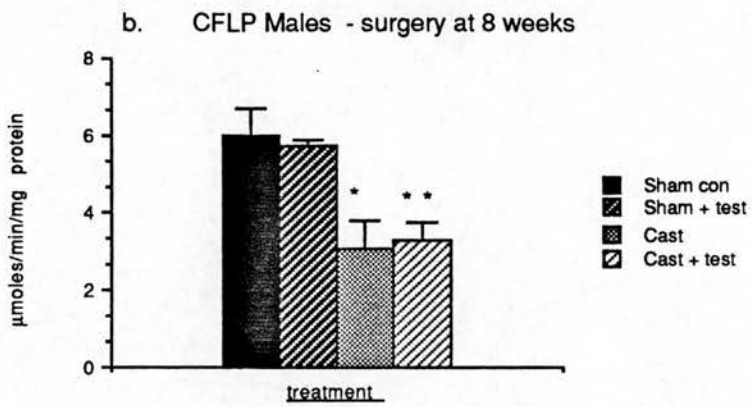
Degrees of significance are assessed relative to sham operated controls in each group.

* $P > 0.01$

** $P > 0.001$

Figure 3.01


a.
CFLP Males - surgery 2 weeks
µmoles/min/mg protein
8
6
4
2
0
* *
*
treatment
Sham con
Sham + test
Cast
Cast + test


b.
CFLP Males - surgery at 8 weeks
µmoles/min/mg protein
8
6
4
2
0
*
* *
treatment
Sham con
Sham + test
Cast
Cast + test

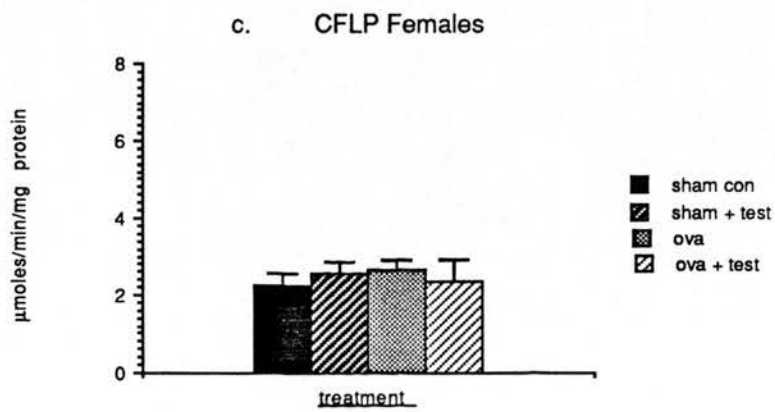

c.
CFLP Females
µmoles/min/mg protein
8
6
4
2
0
treatment
sham con
sham + test
ova
ova + test

Figure 3.02a

Electrophoretic analysis of CFLP males surgically operated upon at 2 weeks of age.

Cytosols (30μg) from individual male CFLP mice sham operated or castrated at 2 weeks of age were analysed by SDS/PAGE on a 12% polyacrylamide resolving gel. The order of samples loaded is as follows : lane 1, rat liver isoenzyme mixtures comprising Yc (Mr 27,500), Yb (Mr 26,300) and Ya (Mr 24,800); lanes 2-5, sham operated controls injected with vehicle; lanes 6-8, sham operated animals treated with testosterone; lanes 9-12, castrated CFLP mice injected with vehicle; lanes13-16, castrated animals treated with testosterone; lane 17, purified mouse Yf.

Figure 3.02a

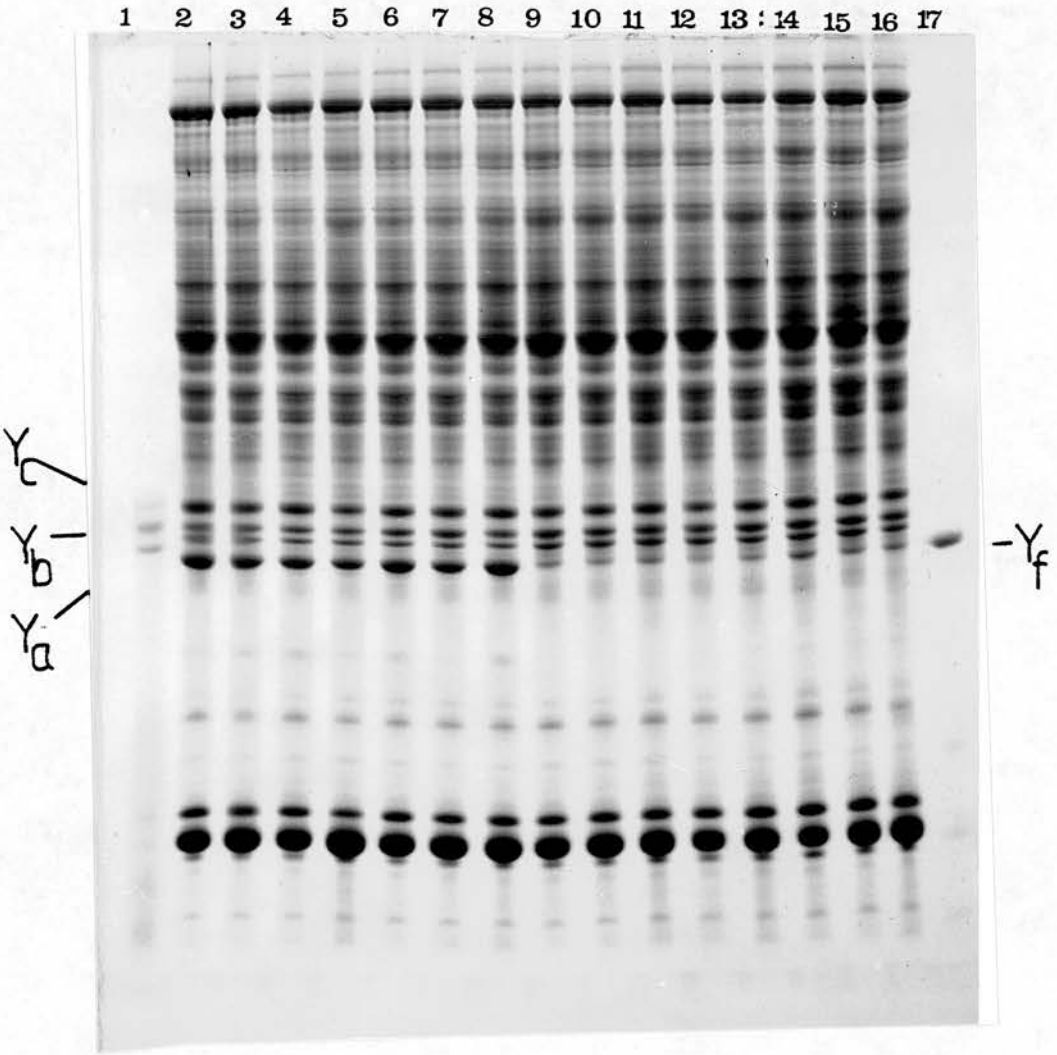


Figure 3.02b

Electrophoretic analysis of CFLP males surgically operated upon at 8 weeks of age.

Cytosols (30μg) from individual mice were analysed by SDS/PAGE on a 12% polyacrylamide resolving gel. Loadings were as follows :- lane 1, rat liver standards (Yc, Yb, Ya), lanes 2-5, sham operated males controls injected with vehicle; lanes 6-9, sham operated males treated with testosterone; lanes 10-11, castrated CFLP mice injected with vehicle; lanes 12-15, castrated animals treated with testosterone; lane 16, purified mouse Yf standard.

Figure 3.02b

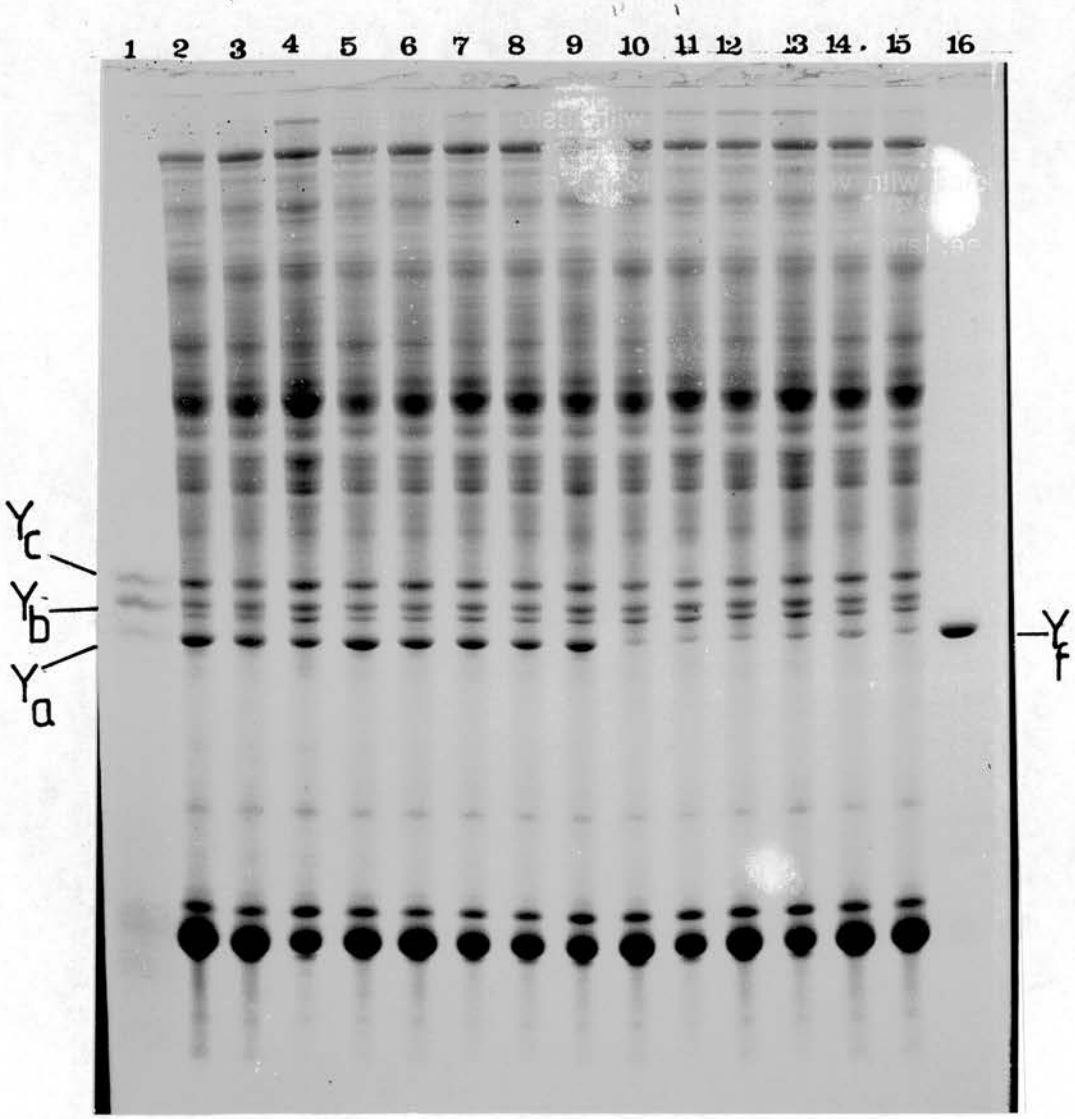
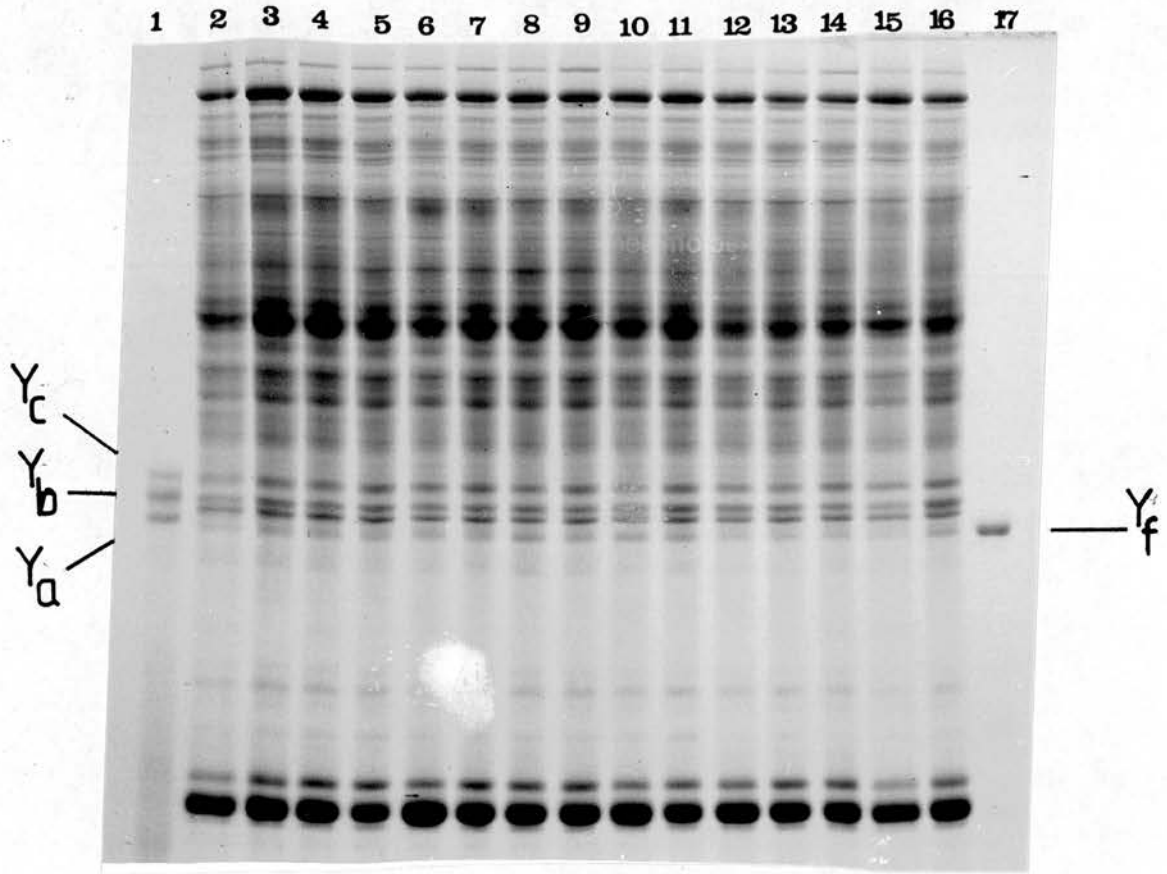


Figure 3.02c

Electrophoretic analysis of CFLP female cytosols.

Cytosols from individual CFLP female mice (30μg), treated as described in section were analysed by SDS/PAGE on a 12% polyacrylamide resolving gel. Gel loadings were as follows :- lane1, rat liver GST isoenzyme mixture (Yc, Yb, Ya); lanes 2-5, sham operated controls injected with vehicle; lanes 6-9, sham operated animals treated with testosterone; lanes 10-13, ovarectomised females injected with vehicle; lanes 14-16, ovarectomised females treated with testosterone; lane 17, purified mouse Yf standard.

Figure 3.02c



alter levels of the Mr 24,800 polypeptide in castrates, although 2 week castrates did show a slight induction. In females, a polypeptide of similar molecular weight was also present, but at much lower levels than observed in males (figure 3.02c). No change in the level were noted either in ovarectomised females or those subjected to testosterone treatment.

Immunoblotting of these samples with anti-mouse Yf antibody mirrored the pattern of expression observed in CDNB activity and the changes noted in the level of the polypeptide of Mr 24,800 (figure 3.03).

It is reasonable to assume that the polypeptide of Mr 24,800, whose expression changes in castrated male mice and which co-migrates with the purified mouse Yf standard, is a Yf-type polypetide similar, if not identical to that isolated and characterised by McLellan and Hayes (1987), since it cross-reacted strongly with monospecific antisera raised against murine YfYf.

3.03 Androgen Regulation of C57BL/6 Mice

3.03a Treatment of Animals

Male C57BL/6 mice were sham operated or castrated at 8 weeks of age. At 11 weeks, the animals were treated either with testosterone propionate, administered subcutaneously at 60μg or 120μg per mouse for 11 consecutive days, or with the vehicle, corn oil. Intact female C57BL/6 mice also received testosterone propionate as detailed above for the males. Table 3.04 summarises the treatment groups obtained and gives the numbers per group.

Figure 3.03a

Immunoblotting of hepatic cytosols from CFLP males castrated at 2 weeks of age using anti-(mouse YfYf) IgG.

Cytosols (50µg protein / track) were subjected to SDS/PAGE followed by transfer to nitrocellulose and immunoblotting with anti-(mouse YfYf)IgG. Gel loadings were as follows; Lanes 1 and 17, purified mouse Yf standard; lanes 2-5, sham operated controls, lanes 6-8, sham operated males treated with testosterone; lanes 9-12, castrated males; lanes 13-16, castrated males treated with testosterone.

Figure 3.03b

Immunoblotting of hepatic cytosols from CFLP mice castrated at 8 weeks of age.

Cytosols (50µg protein / track) were subjected to SDS/PAGE followed by transfer to nitrocellulose and analysed by immunoblotting with anti-(mouse YfYf) IgG. The gel loadings were as follows :- lanes 1,12 and 18, purified mouse liver Yf standard; lanes 2-5, sham operated controls administered vehicle; lanes 6-9, sham operated males treated with testosterone; lanes 10-11, blank; lanes 13-14, castrated males administered vehicle; lanes 15-18, castrated males treated with testosterone.

Figure 3.03c

Immunoblotting of ovarectomised CFLP female mice cytosols using anti-(mouse YfYf) IgG.

Cytosols (50µg protein / track) were subjected to SDS/PAGE, transferred to nitrocellulose and immunoblotted with anti-mouse Yf anti-sera. Loadings were as follows :- lanes 1 and 17, purified mouse liver Yf; lanes 2-5, sham operated controls receiving vehicle; lanes 6-9, sham operated females treated with testosterone; lanes 10-13; ovarectomised females administered vehicle; lanes 14-16, ovarectomised females treated with testosterone.

Figure 3.03

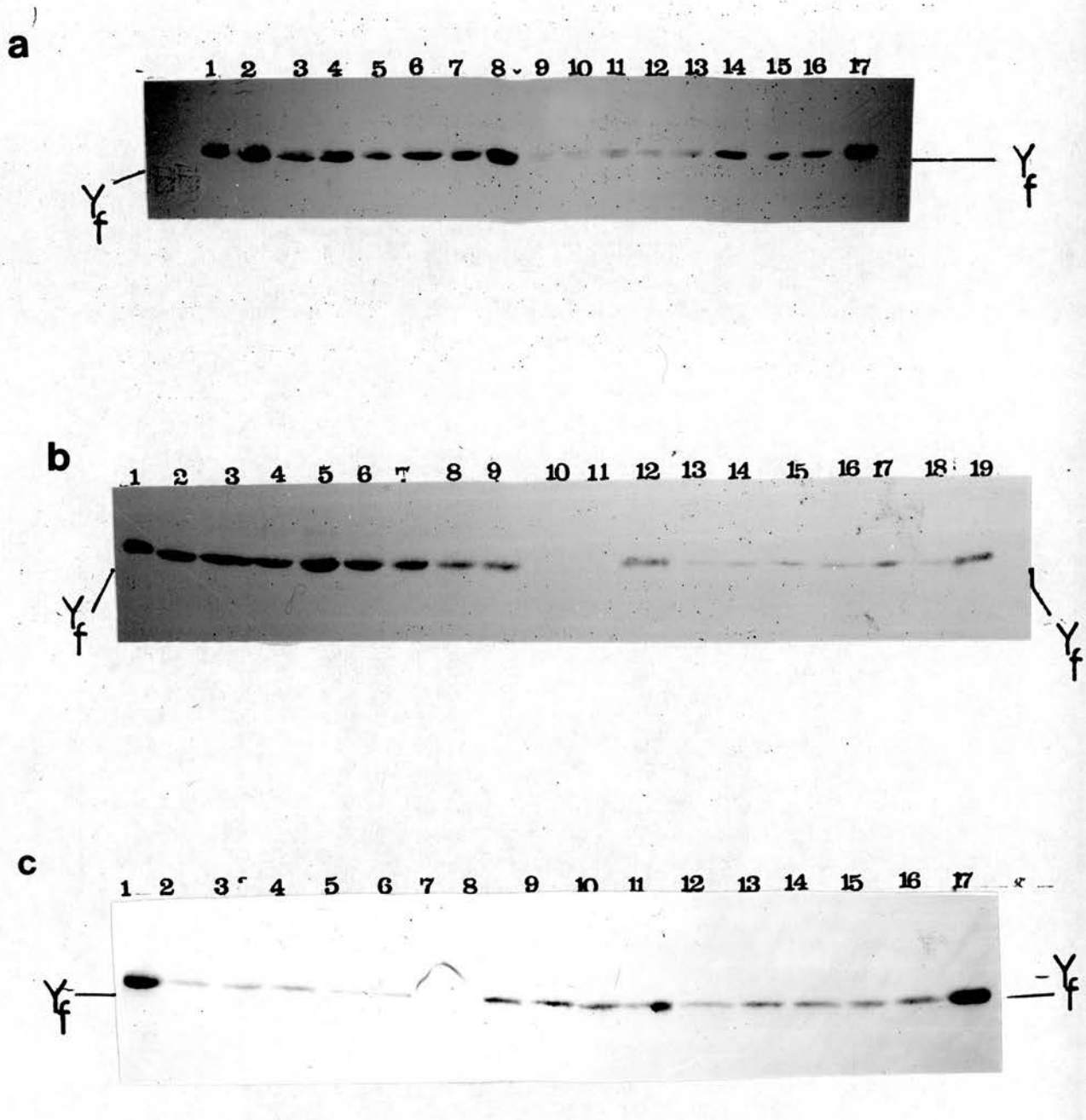

a
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Yf
Yf
b
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Yf
Yf
c
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Yf
Yf

Table 3.04 Treatment groups and numbers for C57BL/6 experiment detailed in in section 3.03a

Group	Treatment	No. per group
Sham operated males	corn oil	5
Sham operated males	testosterone 60µg	4
Sham operated males	testosterone 120µg	4
Castrated males	corn oil	3
Castrated males	testosterone 60µg	4
Castrated males	testosterone 120µg	6
Females	corn oil	4
Females	testosterone 60µg	4
Females	testosterone 120µg	4

3.03b Analysis of Hepatic GST Complement from C57BL/6 Mice

Activity towards CDNB and ethacrynic acid were determined for hepatic cytosols prepared from individual mice treated as described in the previous section. As with the CFLP mice a 2.5-fold difference in CDNB activity between control males and females was observed (figure 3.04). On castration, CDNB activity in the male is reduced towards those levels observed in the females. Administration of testosterone, at both 60μg and 120μg, reverses this decline but does not totally restore levels to those seen in sham male controls. No dose-dependency was observed, testosterone treatment at both concentrations seem to cause a similar induction of activity in castrates. The administration of testosterone to control animals caused no significant change in CDNB activity. Treatment with testosterone induced approximately a 1.5-fold increase in CDNB activity in intact females.

Specific activity measurements towards ethacrynic acid, a GST substrate which is relatively specific for YfYf are presented in figure 3.05. A similar pattern to that observed for CDNB was demonstrated. In the male, castration causes a 5-fold decrease in activity which was partially restored (3-fold) by testosterone treatment. Androgen treatment in the female induced this activity again by approximately 1.5-fold.

Analysis of female hepatic cytosols by SDS/PAGE and immunoblotting using antisera raised against the mouse Yf, Ya_3 and Yb_1 subunits demonstrated that the pattern of Yf expression reflected changes in activity seen with both CDNB and ethacrynic acid. It also seems that this induction was dose-dependent, with 120μg testosterone having a greater effect than 60μg. No change in levels of Ya_3 or Yb-type subunits were detected (figures 3.06 and 3.07).

Figure 3.04. Levels of CDNB activity in hepatic cytosols from a) C57BL/6 male mice undergoing testosterone treatment after gonadectomy and b) intact C57BL/6 females treated with testosterone.

Mean values for all groups are quoted.

Degrees of significance are assessed relative to control values for each sex.

* $P > 0.01$

** $P > 0.001$

Figure 3.04

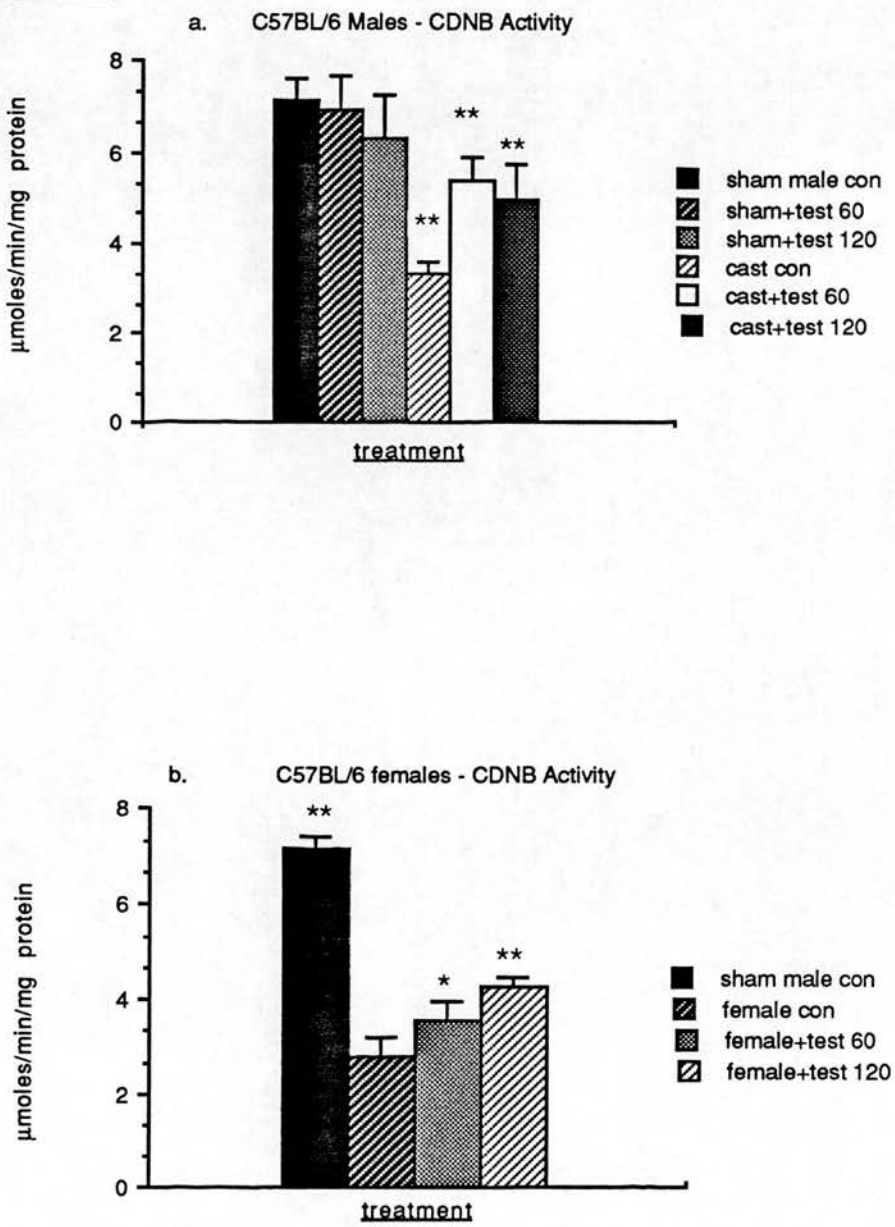

a.
C57BL/6 Males - CDNB Activity
µmoles/min/mg protein
8
6
4
2
0
**
**
**
treatment
sham male con
sham+test 60
sham+test 120
cast con
cast+test 60
cast+test 120
b.
C57BL/6 females - CDNB Activity
µmoles/min/mg protein
8
6
4
2
0
**
*
**
treatment
sham male con
female con
female+test 60
female+test 120

Figure 3.05

Levels of ethacrynic acid activity in a) gonadectomised C57BL/6 males undergoing testosterone replacement therapy and b) C57BL/6 females treated with testosterone.

Mean values for each group are quoted.

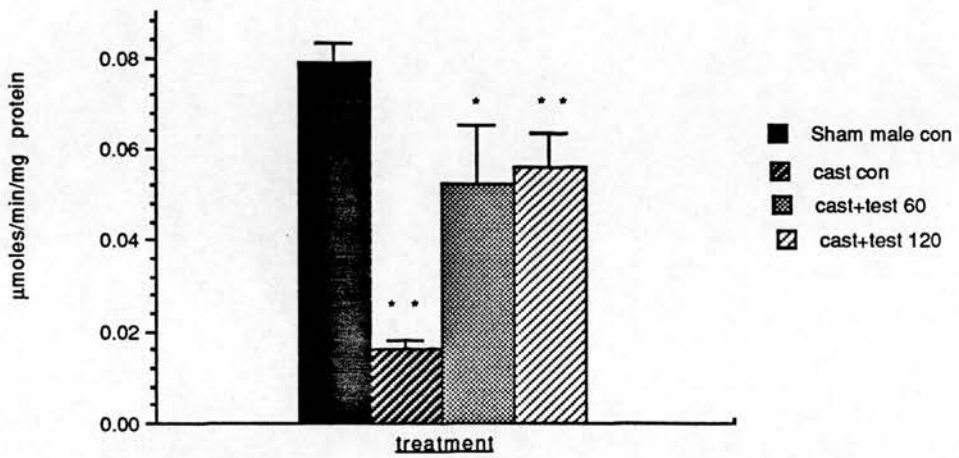
Degress of significance are assessed relative to control values for control males in (a) and control females in (b).

* $P > 0.01$

** $P > 0.001$

Figure 3.05

a. C57BL/6 Males - Ethacrynic acid Activity



b. C57BL/6 Females - Ethacrynic acid activity

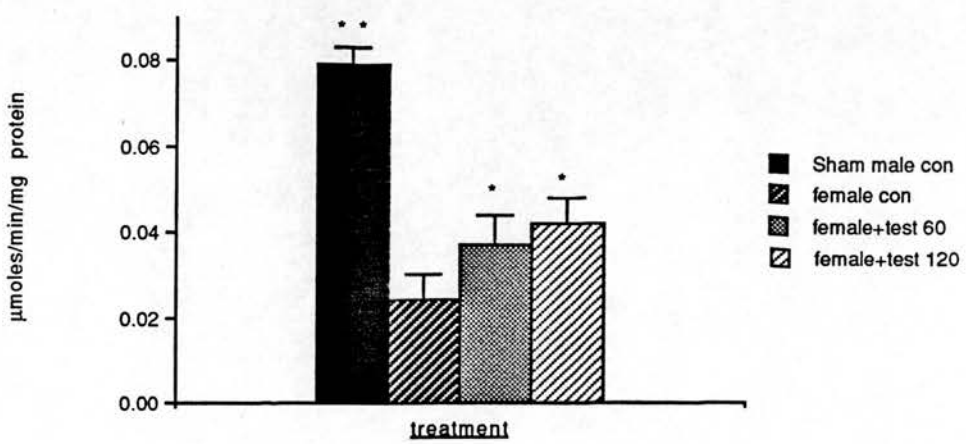


Figure 3.06

Electrophoretic analysis of intact C57Bl/6 female mice treated with testosterone

Hepatic cytosols (30μg protein / track) from individual C57BL/6 females were analysed by SDS/PAGE on a 12% polyacrylamide resolving gel. The loadings were as follows :- lane 1, rat liver isoenzyme mixture (Yc, Yb, Ya); lanes 2-5, females administered vehicle; lanes 6-9, females treated with testosterone at 60μg / day; lanes 10-13, females treated with tesosterone at 120μg / day; lane 14, purified mouse Yf standard.

Figure 3.06

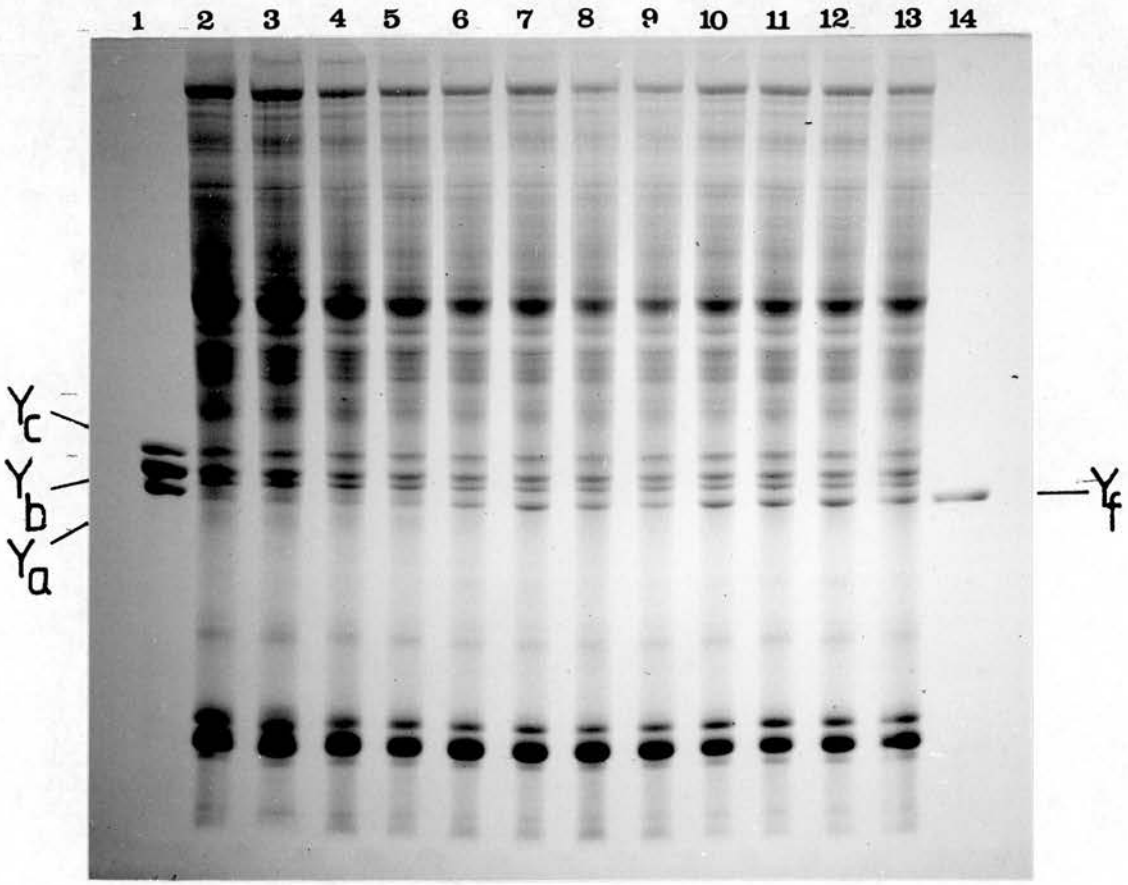


Figure 3.07

Immunoblotting of hepatic cytosols from female C57BL/6 mice treated with testosterone using anti-mouse GST antisera

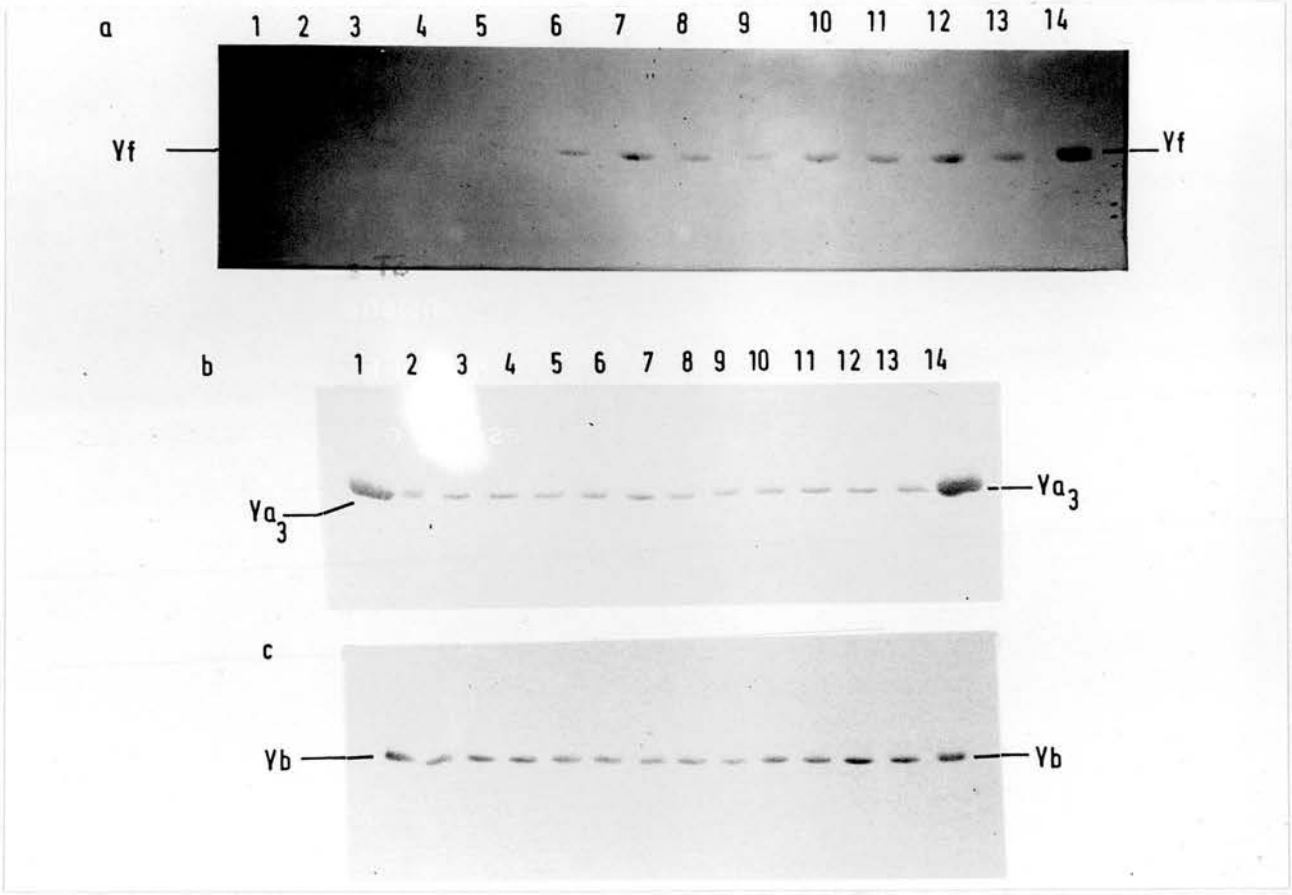
Hepatic cytosols (50μg protein / track) from female C57BL/6 mice treated with testosterone were subject to SDS/PAGE, transferred to nitrocellulose and immunoblotted against anti-mouse GST antisera. Gel loadings were as follows : - lanes1 and 14, mouse GST markers; lanes 2-5, control females administered vehicle; lanes 6-9, females treated with testosterone at a dose of 60μg / day; lanes 10-13, females treated with testosterone at 120μg / day.

Panel a. anti-(mouse YfYf) IgG.

Panel b. anti-(mouse Ya_3Ya_3) IgG.

Panel c. anti-(mouse Yb_1Yb_1)IgG.

Figure 3.07


a
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Yf
Yf
b
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Ya3
Ya3
c
Yb
Yb

Figures 3.08 and 3.09 show the results obtained from SDS/PAGE and western blotting of male cytosolic fraction. The reduction in activities towards the substrates measured above are mainly due to the changes in the Yf GST.

Small changes in the levels of the Ya_3 and Yb-type subunits were observed, however these were not deemed to be significant. The Ya_1 subunit was not detected.

3.04 Characterisation of Hepatic GST Profiles of the Hpg and Tfm Mouse Strains

3.04a. Introduction : A brief description of the Hpg and Tfm defects

The Hypogonadal Mouse (gene symbol hpg)

A mouse mutation has been described, which is inherited in an autosomal recessive fashion, in which the testes and ovaries fail to develop postnatally (Cattenach *et al.* 1977). The primary defect has now been established as a deficiency in the hypothalamic hormone, gonadotropin-releasing hormone (GnRH), which as the name suggests controls the release of the gonadotropins - Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH) from the pituitary gland. The consequent reduction in the levels of these hormones leads to the observed failure in reproductive development. These hormones are of crucial importance in the production and secretion of testosterone from the Leydig cells of the testes. In the male hpg mouse the testes are atrophied and no androgen is produced.

Figure 3.08

Electrophoretic analysis of castrated C57BL/6 males

Cytosols (30μg/track) from individual C57Bl/6 males treated as described in section 3.03a, were analysed by SDS/PAGE on a 12% polyacrylamide resolving gel. The order of gel loadings is as follows :- lane1, rat liver isoenzyme mixture (Yc, Yb, Ya); lanes, 2-4, sham operated animals administered vehicle; lanes 5-7, sham operated animals treated with testosterone at 60μg / day; lanes 8-10, sham operated animals treated with testosterone at 120μg / day; lanes 11-13, castrated C57BL/6 males administered vehicle; lanes 14-16, castrated males treated with testosterone 60μg / day; lanes 17-19, castrated males treated with testosterone at 120μg / day; lane 20, purified mouse Yf standard.

Figure 3.08

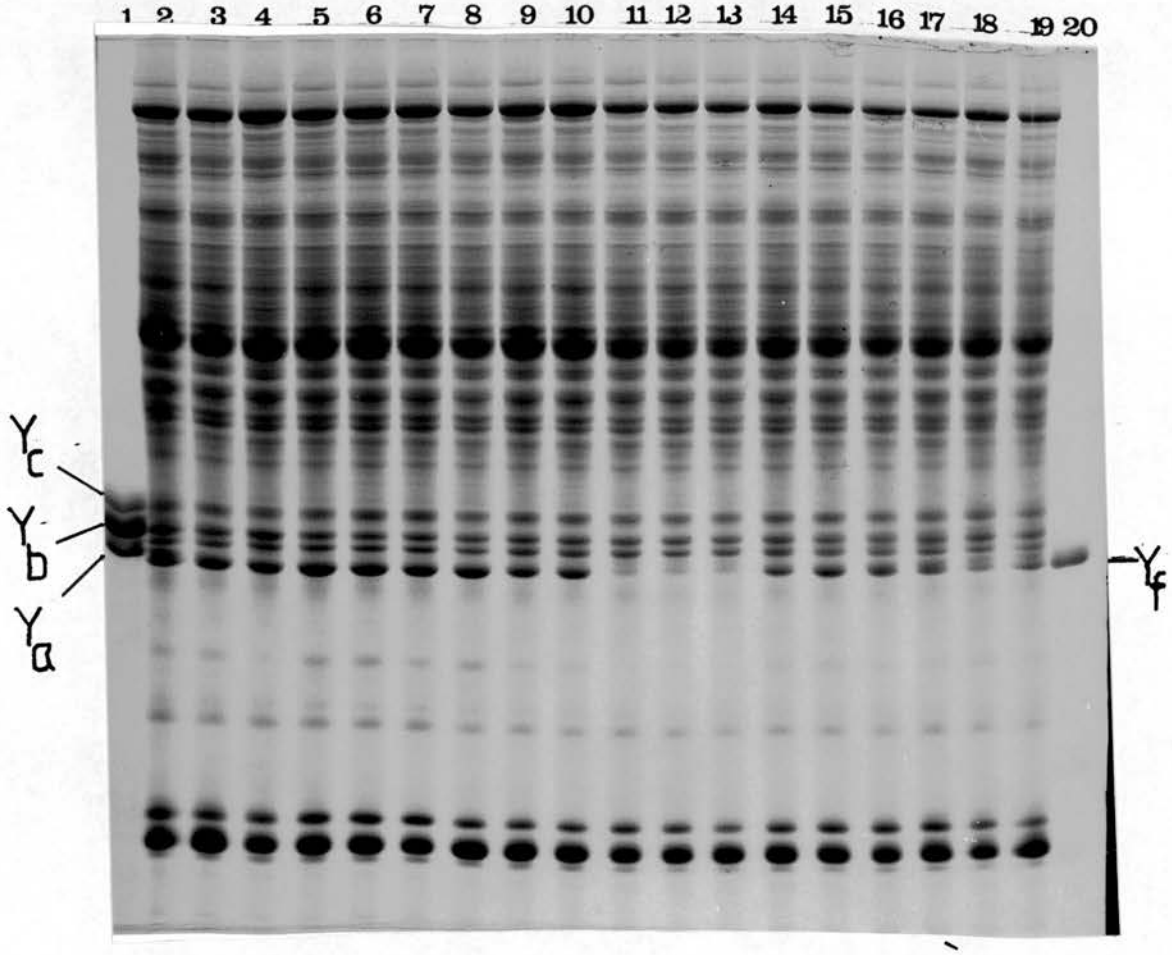


Figure 3.09

Immunoblotting of mouse hepatic cytosols from gonadectomised males under going testosterone replacement therapy

Hepatic cytosols (50μg protein / track) from gonadectomised C57Bl/6 male mice undergoing testosterone replacement therapy were subject to SDS/PAGE, transferred to nitrocellulose and immunoblotted against anti-mouse GST antisera. Gel loadings were as follows :- lanes 1 and 14, mouse GST markers; lanes 2-3, sham operated males administered vehicle; lanes 4-5, sham operated males treated with testosterone at 60μg / day; lanes 6-7, sham operated males treated with testosterone at 120 μg / day; lanes 8-9, castrated males administered vehicle; lanes 10-11, castrated animals treated with testosterone at 60μg / day; lanes 12-13, castrated animals treated with testosterone at 120μg / day.

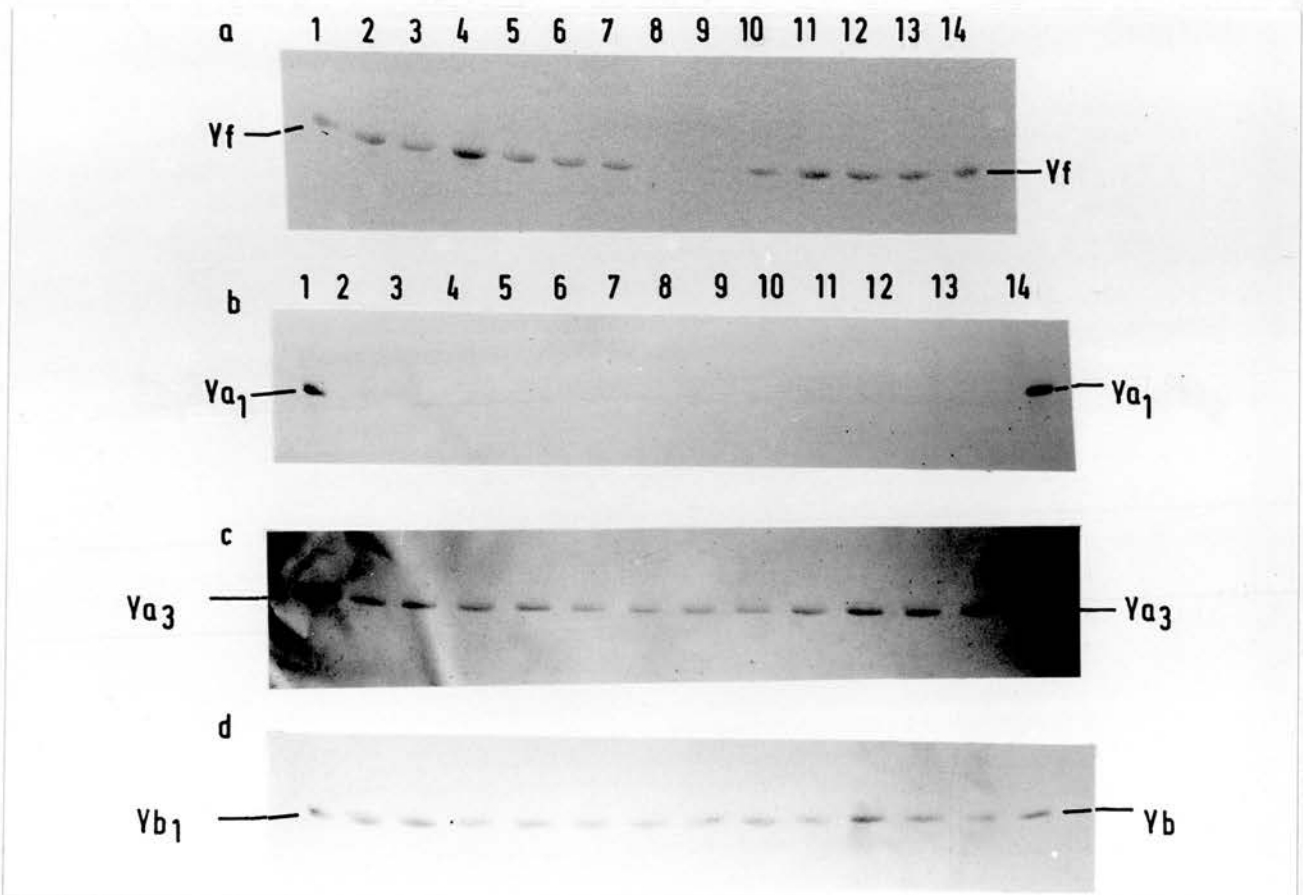
panel a. anti-(mouse YfYf) antisera.

panel b. anti-(mouse Ya_1Ya_1) antisera.

panel c. anti-(mouse Ya_3Ya_3) antisera.

panel d. anti-(mouse Yb_1Yb_1) antisera.

Figure 3.09


a
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Yf
Yf
b
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Ya1
Ya1
c
Ya3
Ya3
d
Yb1
Yb

The Testicular Feminised Mouse (gene symbol tfm)

Testicular feminisation is an X-linked recessive gene defect which has been well documented in Man (Hauser,1963). Affected individuals are male pseudohermaphrodites, characterised by an XY karyotype yet having a female phenotype. The identification of this disorder in rats (Bardin *et al.*, 1970) and mice (Lyon and Hawkes, 1970) led to a clearer understanding of the molecular defect responsible for the impairment of normal sexual differentiation in these males. The underlying genetic lesion has now been established. Reduced or undetectable amounts of the intracellular androgen binding protein in tissues which are normally responsive to androgens has been observed in these mice (Attardi and Ohno,1974; Gehring and Tomkins, 1974). Thus, effects which are normally mediated via this receptor do not occur in these animals.

The hepatic cytosolic GST complement of both mutant strains described above were characterised by the methods used for the previous two sections. The progenitor strain, CH3/He, was used as an appropriate control for the hpg mouse. Both sexes were investigated, with 2 mice per group. Male mice carrying the tfm gene (X^{tfm} Y) were analysed together with wild-type male siblings (XY), three mice were obtained per group.

3.04b Analysis of the Hepatic GST Profile of Hpg and Tfm mice.

Hpg males were found to exhibit a female pattern of expression in respect to their hepatic GST profile. These mutants expressed the Yf subunit in similar amounts to that found in both the normal and mutant females. Levels of the Ya_3 and Yb-type

subunits did not appear to vary greatly between strains and sexes (figure 3.10 and 3.11). This is confirmed using the marker substrates for GST isozymes, activity measurements for which are given in table 3.05. CDNB activity in the hpg male liver was similar to that found in females. Specific activities towards ethacrynic acid were slightly lower in both males and females of the hpg strain than found in normal females.

Table 3.05 Levels of GST activity in hepatic cytosols from male and female the CH3/He and hpg mouse strains. Data from individual mice are presented. Each sample was assayed in triplicate.

		Specific Activity µmoles/min/mg protein				
Strain	Sex	CDNB	DCNB	Ethacrynic acid	CuOOH	H_2O_2
CH3/He	Male 1	4.5	0.06	0.06	1.62	1.17
	Male 2	5.0	0.06	0.04	1.58	1.18
CH3/He	Female 1	2.7	0.05	0.01	1.42	1.07
	Female 2	2.4	0.05	0.01	1.42	1.07
Hpg	Male 1	2.6	0.06	0.003	1.69	1.12
	Male 2	2.3	0.06	0.005	1.53	1.04
Hpg	Female 1	2.5	0.06	0.008	1.70	1.06
	Female 2	3.3	0.09	0.005	1.54	0.92

Figure 3.10

Electrophoretic analysis of hepatic cytosols from the hpg and CH3/He strains of mice

Hepatic cytosols (30μg protein/track) were analysed by SDS/PAGE using a 12% polyacrylamide resolving gel. The loadings were as follows :- lanes 1, rat liver standards (Yc, Yb, Ya); lanes 2-3, CH3/He male mice; lanes 4-5, CH3/He females; lanes 6-7, hpg males; lanes 8-9, hpg females; lane 10, purified mouse Yf standard.

Figure 3.10

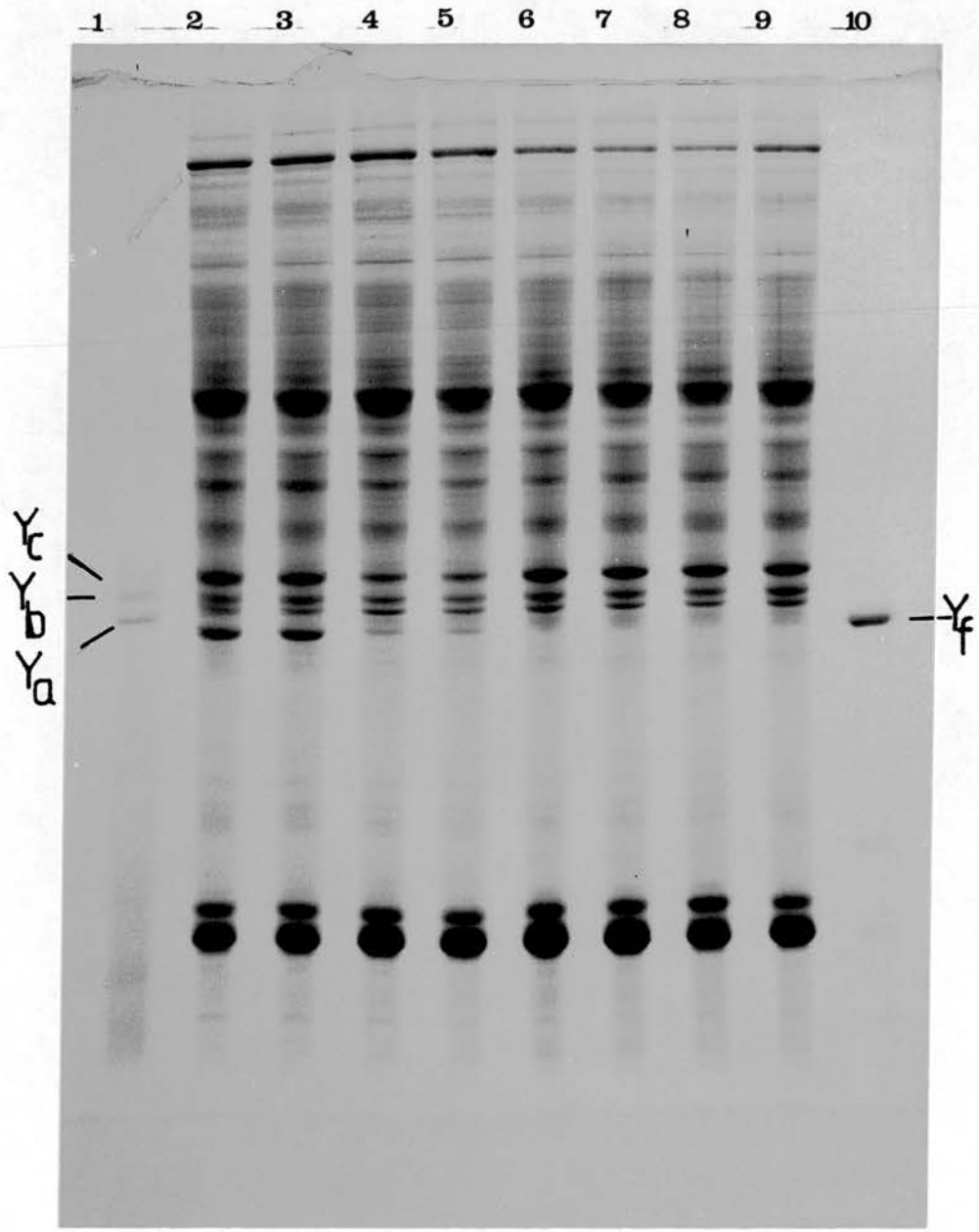
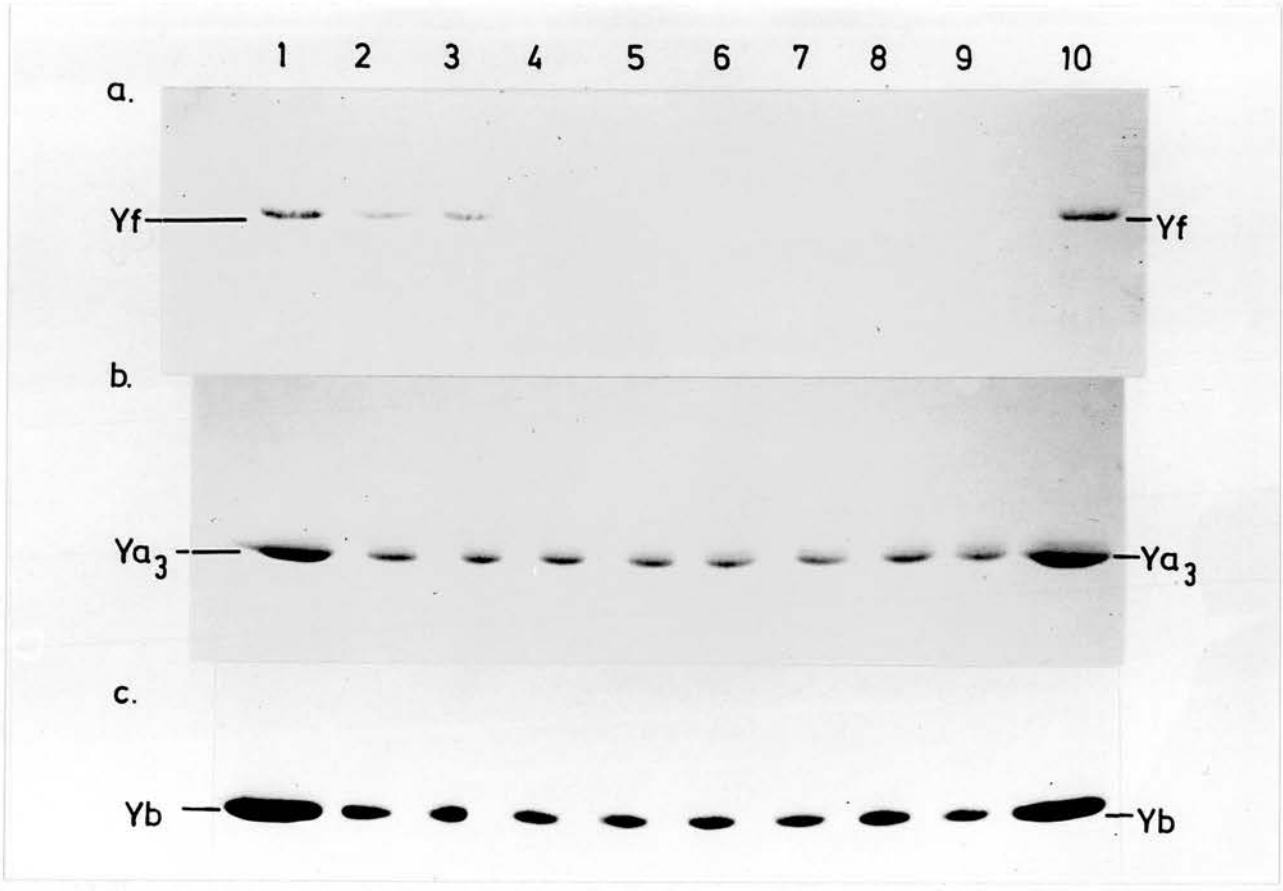


Figure 3.11

Immunoblotting of hepatic cytosols from hpg and CH3/He strains of mice

Hepatic cytosols (50μg of protein/track) were subjected to SDS/PAGE, transferred to nitrocellulose and imunoblotted with antisera raised against purified mouse GST subunits. Gel loadings were as follows :- lanes 1 and 10, purified mouse GST standards; lanes 2-3, CH3/He males; lanes 4-5, CH3/He females; lanes 6-7, hpg males; lanes 8-9, hpg females.

Figure 3.11



Analysis of the tfm mutant was undertaken using the same procedures. A very similar profile to the hpg male was obtained as is shown in figures 3.12 and 3.13. Normal siblings (XY) demonstrated a normal male pattern of expression, whereas males carrying the tfm gene defect showed a feminine phenotype in respect to their hepatic GST profile. Changes in the other GST subunits investigated were not observed. Activity measurements for GST marker substrates were also determined and again showed a very similar pattern to that seen for the hpg males (table 3.06).

Table 3.06 Levels of activity in hepatic cytosols from Tfm males ($X^{tfm}Y$) and their wild-type siblings (XY). Data presented from individual mice. Each sample was assayed in triplicate.

Specific Activity μmoles/min/mg protein

Karyotype	CDNB	DCNB	Ethacrynic Acid	CuOOH	H_2O_2
Male (XY) 1	5.1	0.08	0.08	2.06	1.49
Male (XY) 2	5.8	0.09	0.10	2.06	1.44
Male (XY) 3	5.6	0.07	0.08	1.96	1.44
Male ($X^{tfm}Y$) 1	3.7	0.07	0.04	2.26	1.62
Male ($X^{tfm}Y$) 2	3.5	0.06	0.03	2.28	1.62
Male ($X^{tfm}Y$) 3	3.6	0.08	0.02	2.06	1.45

Figure 3.12

Electrophoretic analysis of hepatic cytosols from Tfm males and control male siblings.

Cytosols from individual mice (30μg protein/track) were analysed by SDS/PAGE on a 12% polyacrylamide resolving gel. The gel was loaded as follows :- lane 1; rat liver isoenzyme mixture (Yc, Yb, Ya); lanes 2-4, control male siblings; lanes 5-7, tfm males; lane 8, purified mouse Yf standard.

Figure 3.12

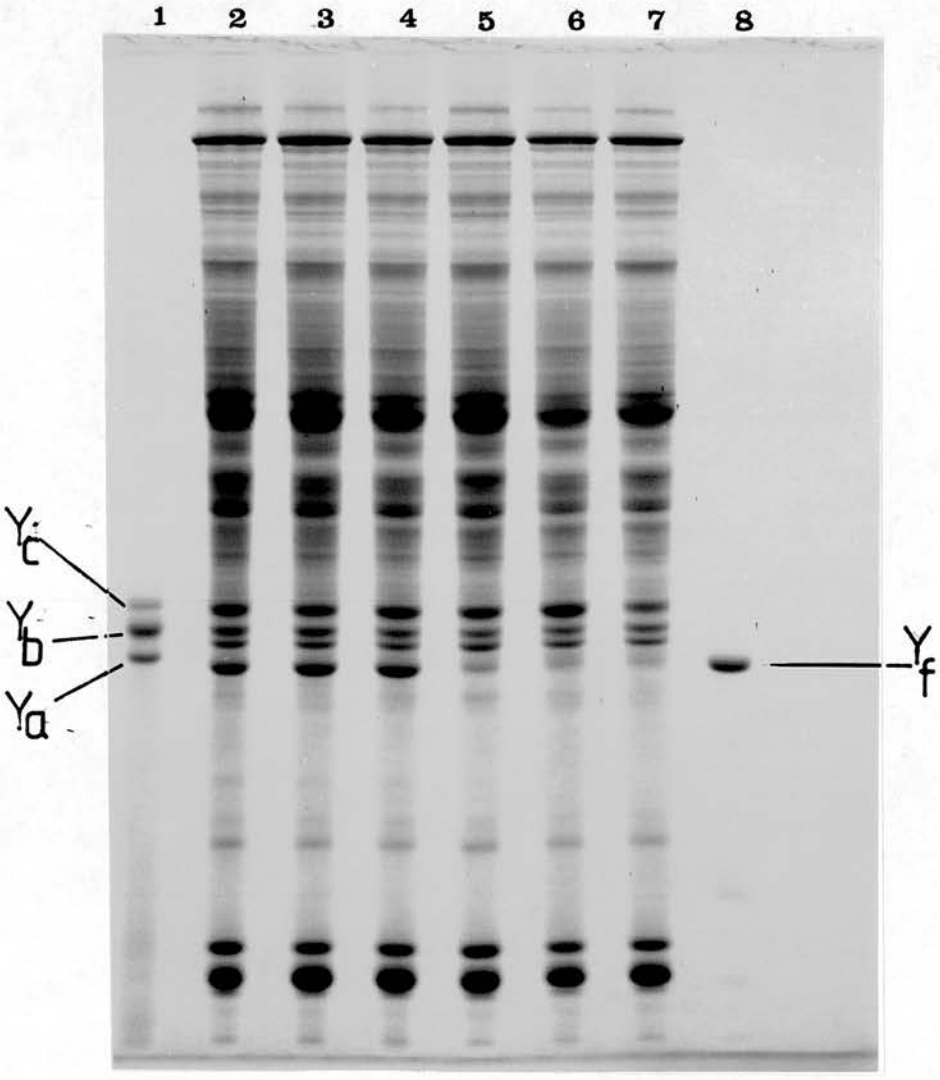


Figure 3.13

Immunoblotting of hepatic cytosols from control and tfm males using anti-mouse GST antisera.

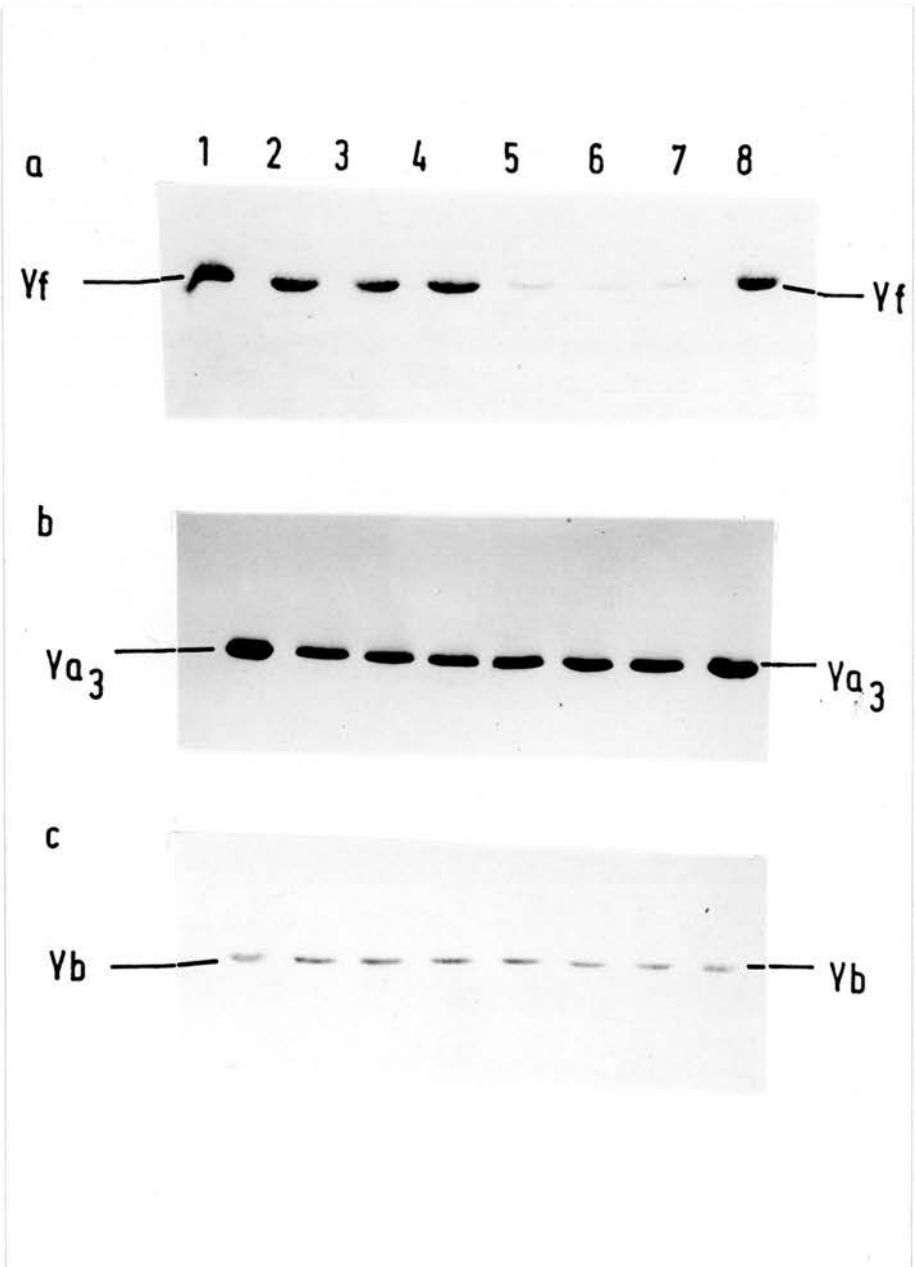
Hepatic cytosols (50μg) were subject to SDS/PAGE after which proteins were electrophoretically transferred to nitrocellulose and immunoblotted against anti-mouse GST antisera. The gel was loaded as follows :- lanes 1 and 8, purified mouse Yf standard; lanes 2-4, normal male siblings; lanes 5-6, tfm males.

Panel a. anti-(mouse YfYf) IgG.

Panel b. anti-(mouse Ya_3 Ya_3) IgG.

Panel c. anti-(mouse Yb_1Yb_1) IgG.

Figure 3.13



3.05 Discussion

The Yf GST subunit is subject to a marked degree of sexual differentiation in the mouse liver, this subunit being the predominant form in the male while only a minor form in the female. This has been characterised very thoroughly by other workers (Hatayama *et al.*, 1986, McLellan and Hayes, 1987). All wild-type strains investigated during the course of this work, CFLP, C57BL/6 and CH3/He, reconfirmed these findings.

Castration of the male reduced levels of Yf to those observed in the female. Replacement therapy with testosterone reversed this effect in C57BL/6 mice, but complete restoration of male Yf GST levels did not occur. Testosterone at both concentrations seemed to produce similar effects, no dose-dependency was observed. Treatment of intact C57BL/6 female mice with androgen caused an elevation in Yf levels in the female liver. A dose-dependent increase was observed, testosterone at 120µg/mouse produced a greater effect than when half that dose was administered. This induction of YfYf in the female liver did not approach levels observed in control males, both CDNB and ethacrynic acid activities were approx 2-fold lower in treated females.

This work confirmed the findings of Hatayama *et al.* (1986) who obtained similar results using the BALB/c mouse strain. Castration reduced YfYf levels in the male to that seen in females. Treatment of intact BALB/c females with testosterone was shown, by radial immunodiffusion with anti-mouse Yf antisera, to cause an elevation in the Yf to male levels. This induction in the BALB/c females is greater than that observed for the C57BL/6 strain, where even in the male complete restoration of the Yf phenotype was not observed. There are a few possible explanations for this

differential induction. The duration of treatment could perhaps have been a factor. Hatayama *et al.* (1986), investigating the time course of induction, showed that levels of the Yf subunit, in the BALB/c females reached a plateau approximately 14 days after treatment started. Females of the C57BL/6 strain were subjected to 11 consecutive days of androgen treatment. Levels of Yf were, however, far lower than than would be predicted at this particular time point in the BALB/c strain and it is unlikely that a further 3 days treatment would alter levels in the C57BL/6 so dramatically. Perhaps strain differences in sensitivity to testosterone or its metabolism could account for this difference in induction between the females, but it is unlikely that males of the C57BL/6 strain would be less sensitive to testosterone than females of another strain. The results obtained for the C57BL/6 strain are, however, more in accord with reports concerning other drug-metabolising systems. Females are found to be relatively resistant to the effects of androgens, they require larger doses and a longer duration of treatment to stimulate the male pattern of expression and even then levels do not completely rise to those observed in the male (Kato, 1968; Berg and Gustafsson, 1973).

It would appear from the results discussed above that androgens regulate the male-specific expression of the Yf GST isoenzyme in mouse liver. Further confirmation for this comes from information obtained from the hpg and tfm strains. Male tfm and hpg mice were found to exhibit a female phenotype in respect to their GST profile. As has already been detailed in section 3.04, in the hpg mice the testes are in an undeveloped state and do not produce testosterone. Plasma testosterone levels in tfm mice are also lower than normal mice (Goldstein *et al.*,1972). However unlike the hpg mouse, which can be rescued by treatment with androgens or with LH and FSH (Ward, 1980), neither testosterone or its androgenic derivatives have any effect on functions which are normally extremely responsive to androgens in the tfm male

(Ohno and Lyon, 1970, Dofuku *et al.*, 1971). It is not entirely clear if this very noticeable decline in Yf levels is due to the low serum levels of testosterone or the absence of its intra-cellular binding protein. In this context, it should be noted that not all androgen-regulated effects occur via the androgen receptor. Paradoxically, some androgen-regulated processes occur via the oestrogen receptor (Gustafsson and Stenberg, 1976b; Bardin and Catterall, 1981) .

Hatayama *et al.*, (1986) also showed that the Yf was developmentally regulated, levels in the immature male being very low and rising rapidly at the onset of puberty, co-inciding with the production and secretion of high levels of testosterone from the testes. These observations further suggest that testosterone essentially regulates expression in the mouse liver of the Yf subunit.

Castrated CFLP mice failed to respond to testosterone. The explanation for this discrepancy probably lies in the treatment regimes which differed between the investigations. C57BL/6 mice were injected with a higher dose of testosterone for a longer duration, i.e. in body weight terms C57BL/6 mice received either a 2mg/kg or 4mg/kg dose for 11 days. CFLP mice received a primary dose of 2mg/kg followed by 6 days at 1mg/kg with a 2 day break after day 5. Two observations provide evidence to this effect. 1. CFLP mice castrated at 2 weeks did show slightly elevated levels of Yf after treatment with testosterone, although no effect was observed for the 8-week castrates or in the females, this would suggest that this particular dose might produce marginal effects on the Yf subunit. 2. Mouse kidney exhibits a sexually differentiated phenotype with respect to P-450 expression, which is directly mediated via testosterone (Henderson *et al.*, 1990). Analysis of kidney microsomes from these CFLP mice demonstrated that castration leads to female pattern of P-450 expression. Treatment of castrates with testosterone partially

restores the male phenotype but superimposed upon this is the female pattern of expression. (C.Henderson, personal communication). This would suggest that the dose administered is not high enough or of sufficient duration to completely restore the masculinised pattern of expression or suppress that of the female.

Ovarectomy of female CFLP mice had no effect on GST levels. It would appear, therefore, that the female sex hormones do not have an inhibitory role to play in the down regulation of Yf in the female. This is in accord with reports on other liver proteins subject to sexual dimorphism. Oestrogens and progestins do not have a large role to play in the generation of this differentiated phenotype. Androgens are the main regulators in this system (Skett, 1987).

Imprinting of the Yf GST

One of the key questions in the regulation of the Yf is its imprintability. Is this enzyme subject to neonatal programming as has been demonstrated for other drug metabolising enzymes? Unfortunately, this is not at all clear from the evidence acquired to date. Most drug and steroid metabolising enzymes conform to the classical definition of imprinting, described in section 3.01b. Gustaffson, however, was able to categorise steroid metabolising P-450s into 3 groups according to their imprinting characteristics (Einarsson *et al.*, 1973; Gustafsson and Steinberg, 1974a,b; Skett and Gustafsson, 1979). These are listed below:

Group 1 - found to be dependent on the continuous presence of androgen to maintain male levels of activity. Both neonatal and postpubertal castration abolished sex differences. The basal level of expression was unaffected by imprinting.

Group 2 - affected by postpubertal castration but enzymic levels did not drop completely to female levels. Neonatal castration was required to generate a complete feminisation in this group.

Group 3 - expression was totally independent of androgens.

From the description of the 3 groups, GST YfYf, if imprinted, would appear to be more closely related to those imprinted enzymes described in group 1. As has been demonstrated, male-specific expression of Yf is androgen dependent. It does not however follow the classical description of the imprinted, group 2 enzymes described by Gustafsson. On castrastion, the level of Yf falls completely to that of the female rather than the partial drop seen for the imprinted group 2 steroid-metabolising enzymes.

Group 1 enzymes are also subject to imprinting although it is of a more subtle form. Neonatal androgens are still required to instigate the male pattern of expression. Thus, if castration occurs postpubertally the effects can be readily reversed by androgen treatment. After neonatal castration however, group 1 enzymes cannot be induced by androgens. Groups can be further subdivided into classes categorised by their neonatal sensitivity to androgens and the time taken to complete the imprinting process.

Ideally in the CFLP experiment, if imprinting of Yf occured, neonatal castration would result in a feminine GST phenotype which would not be effected by testosterone treatment. As has already been discussed, the dose of testosterone administered was too low to restore Yf levels in CFLP castrates (2 or 8 week). Gustafsson and his

colleagues noted that in many cases the imprinting process is completed within 7 days of birth (Gustafsson and Stenberg, 1974, a, b). Thus, 2 week neonatal castrates would probably have been imprinted and as readily inducible with testosterone as those mice castrated at 8 weeks. Neonatal castration should probably be performed in the first few days after birth.

This chapter has established that the Yf subunit is regulated by the male sex hormone testosterone although no firm conclusions can be drawn as to its imprintability. The question now raised is whether this hormone is acting directly on the liver to regulate levels of the Yf or whether the effect is indirect and is mediated via the pituitary. Chapter 4 seeks to address this question.

Chapter 4 - Pituitary Regulation Of The Hepatic Yf GST

4.01 Introduction

4.01a The Role of the Pituitary Gland In Hepatic Sexual Dimorphism

Having established that androgens are responsible for the generation and regulation of sexual dimorphism in a number of liver functions, the question arose as to the mechanism(s) whereby this phenomenon was achieved. That the pituitary gland has a major role to play in the aforementioned process was first demonstrated by Denef (1974) who showed that removal of the pituitary gland (hypophysectomy) abolished sex differences between male and female rats. Denef suggested that the pituitary was acting in a permissive manner to allow androgen secretion. However, hypophysectomised animals were found to be resistant to androgens in respect to hepatic drug and steroid metabolism. In contrast to castrated animals, administration of androgens did not restore the normal sexually differentiated pattern in these animals. These findings indicated that the pituitary was in some manner mediating the effects of androgens on the liver (Gustafsson and Stenberg, 1974c, 1976a; Kramer and Colby, 1976; Wilson and Spelsberg, 1976).

Attempts to reverse the effects of hypophysectomy by the implantation of the pituitary gland under the kidney capsule resulted in a feminine pattern of drug and steroid metabolism in both male and female rats (Denef, 1974; Gustaffson and Stenberg, 1976a, Eneroth *et al.*, 1977). The same effect was also noted in intact animals of both sexes. From these observations, it was postulated that the implanted pituitary was secreting a feminising factor, otherwise referred to as "femotropin". Several laboratories attempted to identify the pituitary hormone responsible for this

effect. Table 4.01 lists the hormones produced by the pituitary, their known functions and their location within the gland.

Several lines of enquiry were pursued in the attempt to answer this question, including the use of implanted, cloned pituitary tumours. Differential secretion of pituitary hormones between isolated tumour types, it was hoped, would narrow down the list of possible pituitary factors. For example, the MtT/F4 tumour secretes prolactin, growth hormone, and ACTH (Bates *et al.*, 1962). When implanted into rats, a feminisation of hepatic steroid metabolism is observed. The MtT/Ws, MtT/W10 and MtT/W15 tumours produce growth hormone and prolactin but little ACTH. This tumour has a similar effect to the MtT/F4 tumour.(Eneroth *et al.*, 1976)

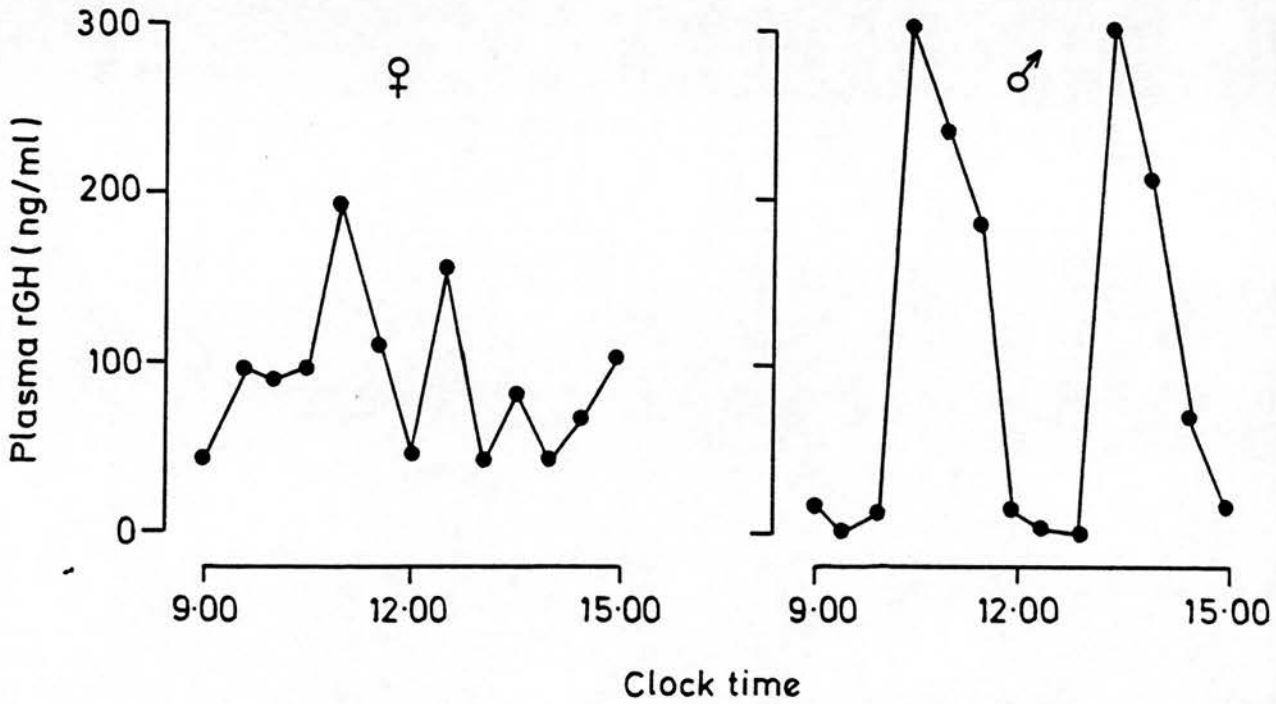
Purified hormone preparations have also been tested for activity. Growth hormone was found to be the most active of the pituitary hormones in influencing steroid metabolism (Colby *et al.*, 1974; Wilson, and Spelsberg. 1976; Kramer and Colby, 1976). The dominant role of growth hormone in the regulation of hepatic drug and steroid metabolism was demonstrated by Mode *et al.* (1981). This was further confirmed when feminising factor was eventually purified and characterised. This factor was found to be identical to growth hormone (Mode *et al.*, 1983)

Eden (1979) demonstrated a very significant difference in the pattern of growth hormone secretion in rat between the sexes. In adult males, strikingly regular, high growth hormone pulses are observed every 3-4 hours, between these peaks, levels are low or undetectable (Fig 4.01). The female rat, on the other hand, displays a more continuous pattern of secretion with higher baselines as compared to the male. This sexually differentiated rhythm of growth hormone release has been shown to regulate sexual dimorphism in the liver. Treatments which stimulate these natural

Table 4.01 Hormones produced by the pituitary gland and a summary of their actions.

Name and Source	Action
Anterior lobe	
Thyroid-stimulating hormone (TSH, thyrotropin)	Stimulates thyroid secretion and growth.
Adrenocorticotropic hormone (ACTH, corticotropin)	Stimulates adrenocortical secretion and growth.
Growth hormone (GH, somatotropin, STH)	Accelerates body growth.
Follicle-stimulating hormone (FSH)	Stimulates ovarian follicle growth in the female and spermatogenises in the male.
Luteinizing hormone (LH, interstitial cell-stimulating hormone, ICSH)	Stimulates ovulation and luteinization of ovarian follicles in female and testosterone secretion in males.
Prolactin (luteotropic hormone, LTH, luteotropin lactogenic hormone, mammotropin)	Stimulates secretion of milk and maternal behaviour.
β-Lipotropin	Involved in stress response.
Intermediate lobe	
α- and β-Melanocyte-stimulating hormones (α- and β-MSH; referred to collectively as melanotropin or intermedin)	Expands melanophores.
Posterior lobe	
Vasopressin (antidiurectic hormone, ADH)	Promotes water retention
Oxytocin	Causes milk ejection.

Figure 4.01 Secretion of growth hormone in male and female rats



patterns of growth hormone secretion in the male (intermittent injection) and in the female (continuous infusion via osmotic mini-pump) influence steroid metabolism in a predictable manner.

P-450 16α (male-specific) and P-450 15β (female-specific) are useful markers to probe the mechanisms which generate sex differences in the liver. In an elegant series of experiments, Gustafsson and his co-workers have shown that these P-450 enzymes are regulated by the growth hormone secretory pattern (for review, see Zaphiropoulos *et al.*, 1989).

Hypophysectomy of female rats leads to a fall in levels of P-450 15β, no change is observed in the male where levels are low. Continuous infusion of growth hormone to normal male animals and hypophysectomised females increased 15β hydroxylase activity towards normal feminine levels. Intermittent injection of growth hormone had no effect. (MacGeoch *et al.*, 1984, 1985). In contrast, P-450 16α is dependent on an intermittent pattern of growth hormone release. Hypophysectomy resulted in a decline in P-450 16α levels. Continuous infusion of growth hormone caused a further decline. Intermittent injection of growth hormone restored P-450 16α to levels observed in the intact adult male. Hypophysectomised females responded in a similar manner (Morgan *et al.*, 1985; Kato *et al.*, 1986)

Treatments which masculinise or feminise drug and steroid metabolism in the liver have also been found to affect growth hormone secretion. Administration of oestrogens to male rats feminises the pattern of growth hormone secretion, androgens masculinise the growth hormone pattern in female rats (Mode *et al.*, 1982). Prolactin, oestrogen (Norstedt,1982), steroid sulfatase (Erikson *et al.*, 1989), epidermal growth factor receptor (Ekberg *et al.*, 1989; Kashimata *et al.*,

1989) and insulin-like growth factor I (Maitler *et al.*, 1988)are all examples of hepatic sexually differentiated proteins which have been found to be under the control of growth hormone secretory pattern .

In the mouse, although less well characterised than the rat, a similar system appears to be operating. Hepatic prolactin receptors, mouse major urinary proteins (MUPs) production (which are similar to α_{2u} globulin in the rat)(Norstedt and Palmiter, 1984), and the metabolism of hexobarbital are regulated by the pattern of growth hormone release (MacLeod and Shapiro, 1989).

Furthermore, neonatal castration in the male results in a feminisation of the growth hormone secretory pattern. Testosterone treatment during the neonatal period partially restores the male pattern, further treatment with testosterone during adulthood fully restores the male pattern of secretion. Thus, in the mechanism generating sexually dimorphic expression in the liver, androgens are responsible for imprinting the male-specific pattern of growth hormone secretion in the neonatal period, which in turn regulates the differences observed in liver proteins subject to sex-specific expression (Jansson *et al.*, 1985; Jansson and Frohman, 1987).

4.01b Pituitary regulation of Yf

From the results obtained in chapter 3, the sexually differentiated mouse hepatic Yf subunit would appear to be under the control of androgens. In light of the findings detailed above for other sexually differentiated systems in the liver, the possible role of the pituitary was investigated with respect to the Yf subunit. Two lines of enquiry were undertaken to examine this question.

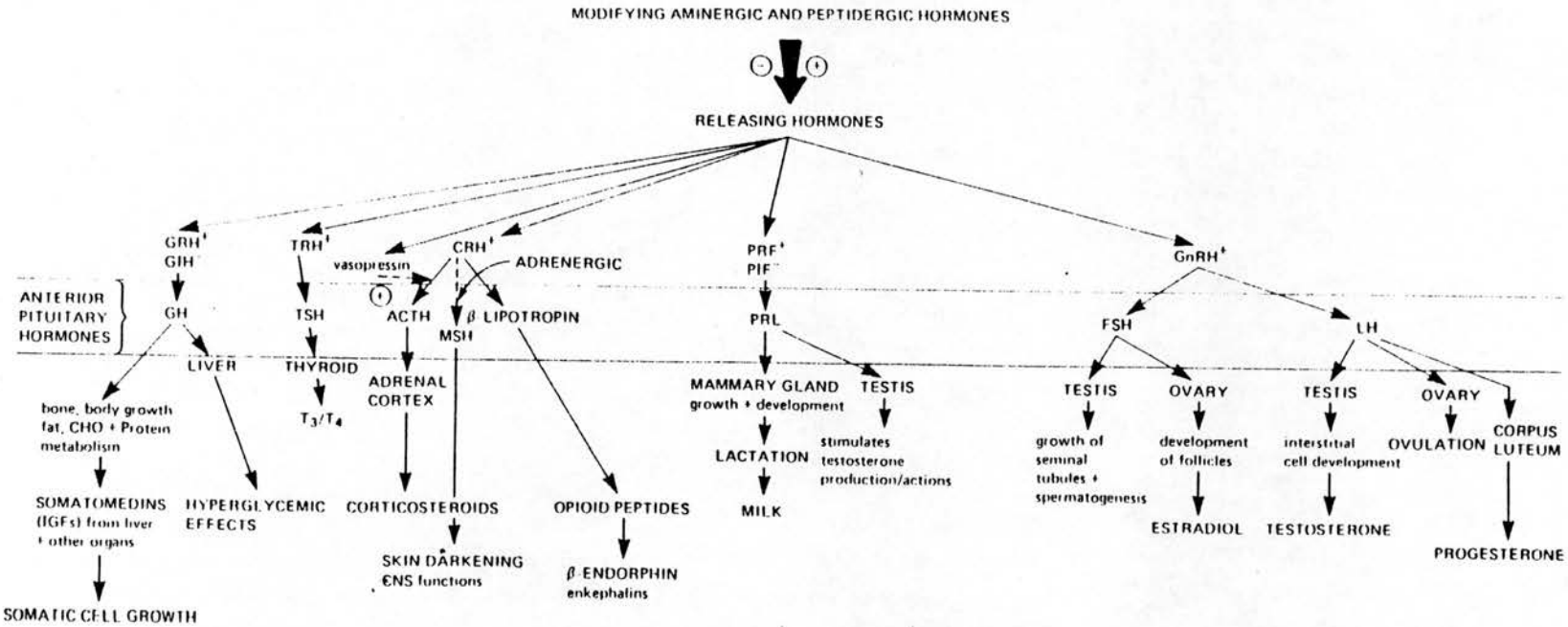
Hypophysectomy

Removal of the pituitary gland has been used as a standard technique to investigate questions of this type. C57BL/6 mice, both male and female, were hypophysectomised and their hepatic GST profile characterised. However, this line of enquiry was not pursued in any great depth for a number of reasons; there were difficulties in obtaining the animals (which had to be transported from the USA after surgery), problems were also encountered due to incorrectly hypophysectomised animals and finally the complexity of the situation makes it very difficult to draw firm conclusions.

The pituitary gland is the main regulator of the endocrine system, itself controlled by factors released from the hypothalamus. Removal of this gland leads to a shut-down of the entire endocrine system. Without the appropriate hormones being released from the pituitary, the adrenals, the thyroid and the gonads all fail to function. Figure 4.02 illustrates the many and diverse processes which are ultimately governed by the pituitary. Thus, for any effect observed it is difficult to ascertain which pathway is actually involved or indeed whether this phenomenon is simply a consequence of the system breaking down.

However, in conjunction with hormone replacement therapy, this animal model can provide very useful information. The advantage here, is that because the entire endocrine system has been "turned off", effects of a single hormone can be examined without the situation being confused by possible interactions with other hormones. Using this technique, it is also possible to determine which hormones are acting directly to regulate the effect in question and which are acting in an indirect or

Figure 4.02 Overview of the anterior pituitary hormones and systems under their control.



Overview of the anterior pituitary hormones showing the connections between the aminergic hormones and neurotransmitters of the CNS, the releasing hormones from the hypothalamus, and the anterior pituitary hormones together with the organs upon which they act and their general effects. GRH, Growth hormone releasing hormone, or somatocrinin; GIH, growth hormone release-inhibiting hormone, or somatostatin; TRH, thyroid-stimulating hormone releasing hormone; CRH, corticotropic releasing hormone; PRF, prolactin releasing factor; PIF, prolactin release-inhibiting factor; GnRH, gonadotropic releasing factor; GH, growth hormone; TSH, thyrotropic-stimulating hormone; ACTH, adrenocorticotropic hormone; MSH, melanocyte-stimulating hormone; PRL, prolactin; FSH, follicle-stimulating hormone; LH, luteotropic hormone; CHO, carbohydrate; IGFs, insulin-like growth factors; T_3, triiodothyronine; T_4, thyroxine. Superscript plus or minus signs or encircled plus or minus signs refer to positive or negative actions.

permissive manner, as has been described for the pituitary regulation of hepatic dimorphism.

The "Little Mouse" Model.

Several mutant mouse strains deficient in a range of endocrine functions are now used quite extensively to investigate hormone-related phenomena. Charlton (1984) provides an excellent summary of the most commonly used strains. Several mutants have now been described which exhibit dwarfism; the Snell dwarf, the Ames dwarf , the "little" mouse, the pygmy mouse, and the hypothyroid mouse. The Snell, Ames and hypothyroid mouse display multiple anterior pituitary hormone deficiencies whereas the "little" mouse has a specific defect in growth hormone synthesis and secretion which makes it an excellent model for the study of growth hormone-regulated processes.

The little mouse mutant, derived from the C57BL/6 strain was first reported by Beamer and Eicher (1976). The defect, inherited in autosomal recessive fashion, was the product of a single gene locus located on chromosome 6. Mice homozygous for this recessive mutation were approximately 60% the size of normal mice and their heterozygote siblings, who display normal growth characteristics. This new mutation was designated "little", gene symbol (lit).

The pituitary growth hormone content in little mice was found to be 4-8% of normal mouse levels (Cheng *et al.*, 1983; Clark and Robinson, 1985). This reduction in growth hormone content was also reflected in growth hormone mRNA levels, which were also reported 8% of normal (Cheng *et al.*, 1983). Other pituitary functions are apparently normal, although there is uncertainty as to the levels of prolactin in the

little mouse. Some workers have reported prolactin as being decreased in comparison to the normal mouse (Beamer and Eicher, 1976)) others have found pituitary prolactin levels to within the normal range (Philips *et al.*, 1982). Table 4.02 gives the pituitary profiles of the Little, Ames and Snell dwarfs.

The genetic abnormality in the production of growth hormone has now been identified. Growth hormone-releasing hormone (GHRH) is a peptide secreted from the hypothalamus which stimulates growth hormone synthesis and secretion from pituitary somatotrophs (growth hormone producing cells). This effect is mediated via a specific receptor on the surface of these cells. In the little mouse, a defect in this receptor confers resistance to GHRH. Thus, growth hormone is neither synthesized or released in response to this signal (Clark and Robinson, 1985; Jansson *et al.*, 1986). The specificity of this defect makes the little mouse an excellent model for the study of growth hormone mediated processes.

4.01c Aim Of This Chapter

This aim of the work contained in this chapter was to investigate the role of the pituitary, with special reference to growth hormone, in the sexual differentiation of the mouse hepatic Yf subunit by : a) investigating the effect of hypophysectomy on mice, both male and female, of the C57BL/6 strain.

b) undertaking a very thorough characterisation of the growth hormone deficient little mouse model.

c) examining the effect of hormone replacement therapy on the little mouse.

Table 4.02 Pituitary profiles for the Snell, Ames and Little Mouse.

Name of mouse gene	Chromosome assignment	Blood hormone status					Mode of inheritance
		GH	PRL	TSH	LH	FSH	
Little (lit)	6	D	N	N	N	N	Autosomal recessive
Ames (df)	11	D	D	D	N	D*	Autosomal recessive
Snell (dw)	16	D	D	D	N	D	Autosomal recessive

GH, growth hormone PRL, prolactin TSH, thyroid stimulating hormone

LH, luteinising hormone, FSH, follicle stimulating hormone

* Male only, females normal.

N - normal levels

D - level down in comparison to wild-type mice.

4.02 Analysis of the Hepatic GST Profile in the Hypophysectomised Mouse

4.02a Animal Treatments

Male and female C57BL/6 mice, obtained from Charles River laboratories, Willmington, Massachusetts, USA, were sham operated or hypophysectomised at 6 weeks of age (four animals per group). Mice were allowed to recover from the operation for 2 weeks before transportation. After transit, mice were acclimatized at the ICRF Clair Hall laboratories in Oxford for two weeks before sacrifice. Animals were killed by cervical dislocation, livers were removed rapidly, frozen and stored at -70°C. Immediately after sacrifice, blood was collected from each mouse, spun down in lithium heparin tubes, the plasma collected and frozen at -70°C.

The sham operated females were unfortunately lost during shipment and had to be replaced with age-matched normal female C57BL/6.

Changes in body weight and measurements of triiodothyronine (T_3) levels, a hormone produced by the thyroid gland, were used to determine the success of the operative procedure. Completely hypophysectomised mice have a stable or gradually decreasing body weight, while incompletely hypophysectomised animals increase in body weight. A decline in plasma T_3 levels also indicated that the procedure had been performed correctly. Figure 4.03 shows changes in body weight over a 2 week period for hypophysectomised males and females with sham operated males as a comparison. A decline in plasma T3 was also noted in the hypophysectomised males. Normal females displayed low levels of T3, which did not change on hypophysectomy (Table 4.03).

It is not known if the low level of T_3 observed in the normal female is due to a sex difference in production and/or metabolism of this hormone between male and female mice. But this finding would suggest that measurement of body weight changes (refer to figure 4.03) would be a more reliable indicator of the success of hypophysectomy in the female.

Figure 4.03

Changes in body weight (g) over a two-week period for hypophysectomised mice of both sexes and sham operated male controls.

Figure 4.03

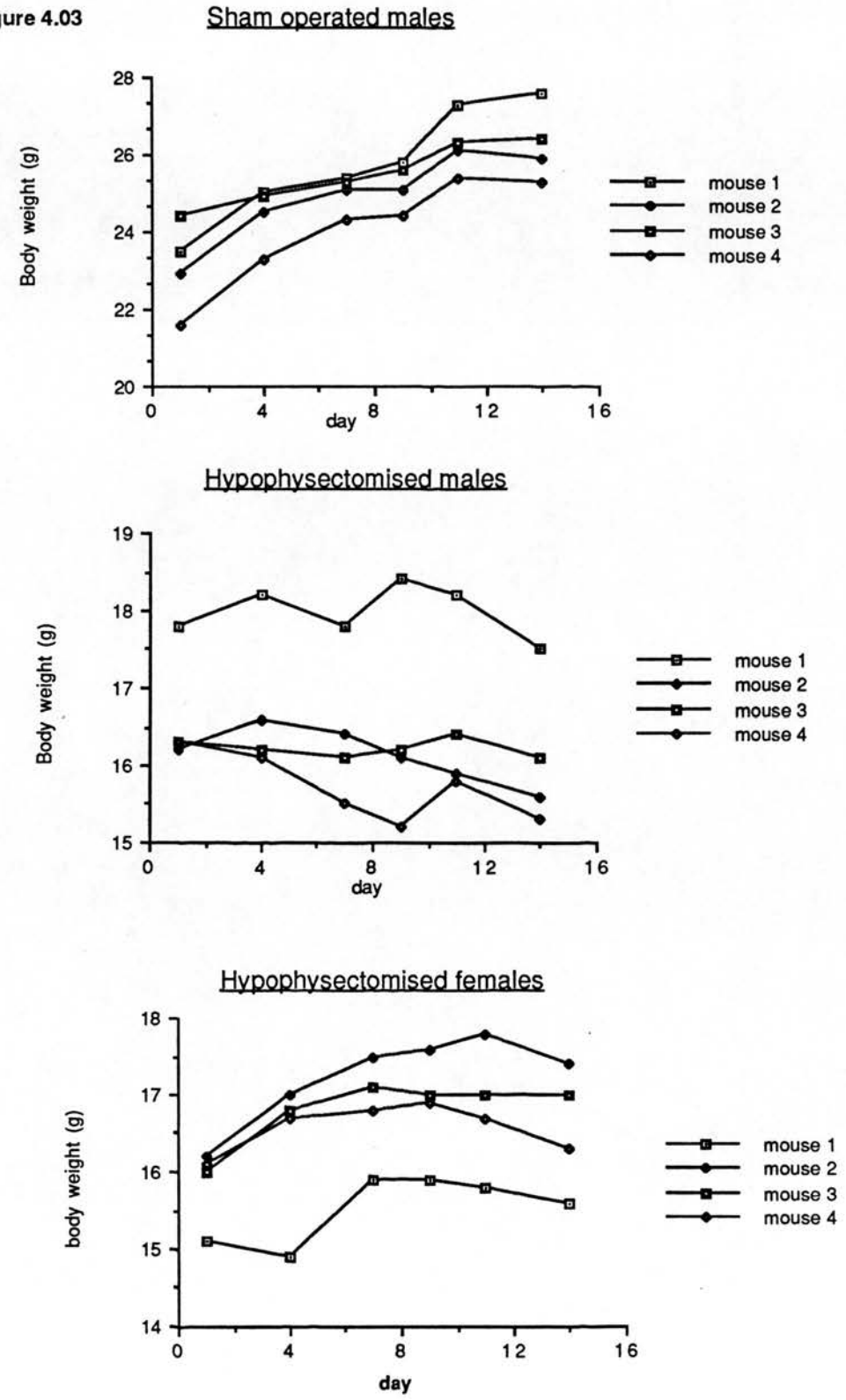

Sham operated males
Body weight (g)
28
26
24
22
20
0
4
8
12
16
day
mouse 1
mouse 2
mouse 3
mouse 4
Hypophysectomised males
Body weight (g)
19
18
17
16
15
0
4
8
12
16
day
mouse 1
mouse 2
mouse 3
mouse 4
Hypophysectomised females
body weight (g)
18
17
16
15
14
0
4
8
12
16
day
mouse 1
mouse 2
mouse 3
mouse 4

Table 4.03 Levels of T_3 in hypophysectomised and control C57BL/6 mice.

Group	Animal No.	Concentration T_3 (nmol/l)
Sham operated males	1	0.947
	2	1.172
	3	1.031
	4	0.921
Hypox males*	1	0.294
	2	0.158
	3	ND
	4	0.117
Normal females	1	0.255
	2	0.530
	3	0.431
	4	0.748
Hypox female	1	0.456
	2	0.370
	3	0.560
	4	0.448

Assays performed in duplicate.

Significance was assessed relative to control of the same sex. * $P > 0.01$

hypox, hypophysectomised; ND, not determined.

4.02b Analysis of the hepatic GST profile from hypophysectomised animals.

Cytosols, prepared from hypophysectomised animals and their controls, were assayed for activity towards a panel of GST substrates, specific activity measurements for which are presented in table 4.04. On removal of the pituitary, specific activities towards CDNB and ethacrynic acid in the male were found to fall to levels observed in the hypophysectomised female, which appear to be slightly elevated as compared to the normal female.

Interestingly, an approximately 2-fold decrease in glutathione peroxidase activity was also noted, both for CuOOH and H_2O_2 (which assay total cellular peroxidase activity and the Se-dependent form respectively). This would suggest that the removal of the pituitary is, by some mechanism, altering (Se-dependent) glutathione peroxidase.

Analysis of hepatic proteins by SDS/PAGE and western blotting with antisera towards mouse GST subunits, demonstrates that on hypophysectomy, a sharp decline in the levels of Yf in the male occurs (Figures 4.04, 4.05). In the female, a slight decrease in Yf levels would appear to occur on removal of the pituitary. This change is also reflected at the mRNA level (figure 4.06).

Interestingly, levels of Yb-type subunits and the Ya_3 would appear to be induced in hypophysectomised animals of both sexes (figure 4.04). This change however is less evident on immunoblotting (figure 4.05b, 405d). The BHA-inducible Ya_1 subunit, which is normally present in low or undetectable amounts in normal mouse liver (McLellan and Hayes, 1989), is markedly induced on hypophysectomy in both sexes (figure 4.05c). This would suggest that, in addition to the Yf GST, the endocrine system is involved in the regulation of other hepatic GST forms.

Table 4.04 Levels of GST activity in hepatic cytosols from hypophysectomised C57BL/6 mice.

Treatment	Sex	CDNB	DCNB	Ethacrynic Acid	CuOOH	H_2O_2
Hypophysectomy	Male	4.7*	0.115	0.023**	1.4	0.76*
		(0.58)	(0.018)	(0.003)	(0.42)	(0.16)
	Female	4.6*	0.111	0.018**	1.4	0.75*
		(0.6)	(0.013)	(0.002)	(0.07)	(0.07)
Sham operated	Male	6.7	0.101	0.063	1.8	1.25
		(0.55)	(0.014)	(0.007)	(0.25)	(0.19)
Normal	Female	3.0*	0.064*	0.014**	1.9	1.45
		(0.16)	(0.01)	(0.001)	(0.071)	(0.077)

Mean values for each group are quoted, * $P > 0.01$

** $P > 0.001$

Degrees of significance are assessed relative to male sham operated controls. Standard deviations are quoted in brackets below mean values. Units - μmoles/min/mg protein.

Figure 4.04

Electrophoretic analysis of hepatic cytosols from both sexes of hypophysectomised and control C57BL/6 mice.

Cytosols from mouse liver (30µg of protein/track) were analysed by SDS/PAGE using a 12% polyacrylamide resolving gel. The gel was loaded as follows :- lane 1, rat liver markers (Yc, Yb, Ya); lanes 2-5, hypophysectomised males; lanes 6-9, hypophysectomised females; lanes 10-13, sham operated males; lanes 14-17, normal females; lane 18, purified mouse Yf.

Figure 4.04

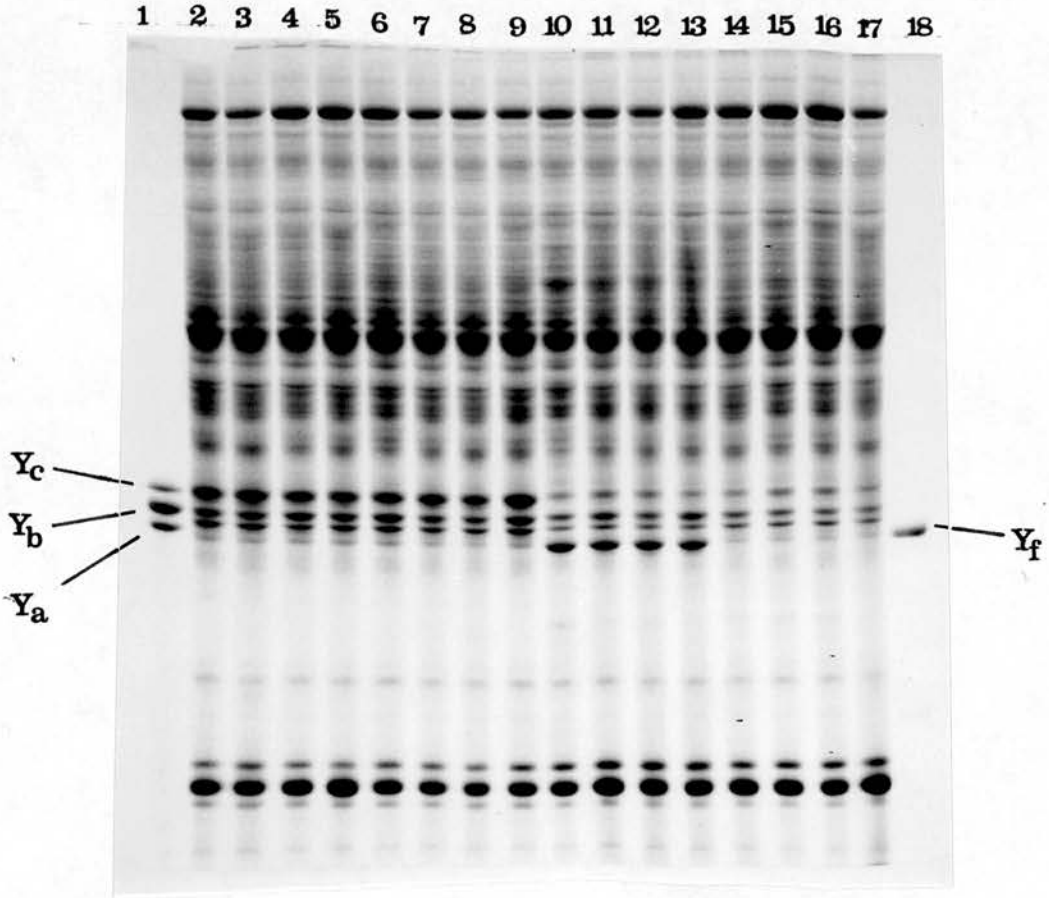


Figure 4.05

Immunoblotting of hepatic cytosols from hypophysectomised and control C57Bl/6 mice of both sexes.

Hepatic cytosols (50μg protein/track) were subjected to SDS/PAGE followed by transfer to nitrocellulose and immunoblotting with antisera against purified mouse subunits. The gel was loaded as follows :- lanes 1 and 14, GST markers; lanes 2-4, hypophysectomised males; lanes 5-7, hypophysectomised females; lanes 8-10, sham operated males; lanes 11-13, normal females.

Panel a. anti-(mouse YfYf) IgG.

Panel b. anti-(mouse Yb_1Yb_1)IgG.

Panel c. anti-(mouse Ya_1Ya_1)IgG.

Panel d. anti-(mouse Ya_3Ya_3)IgG.

Figure 4.05

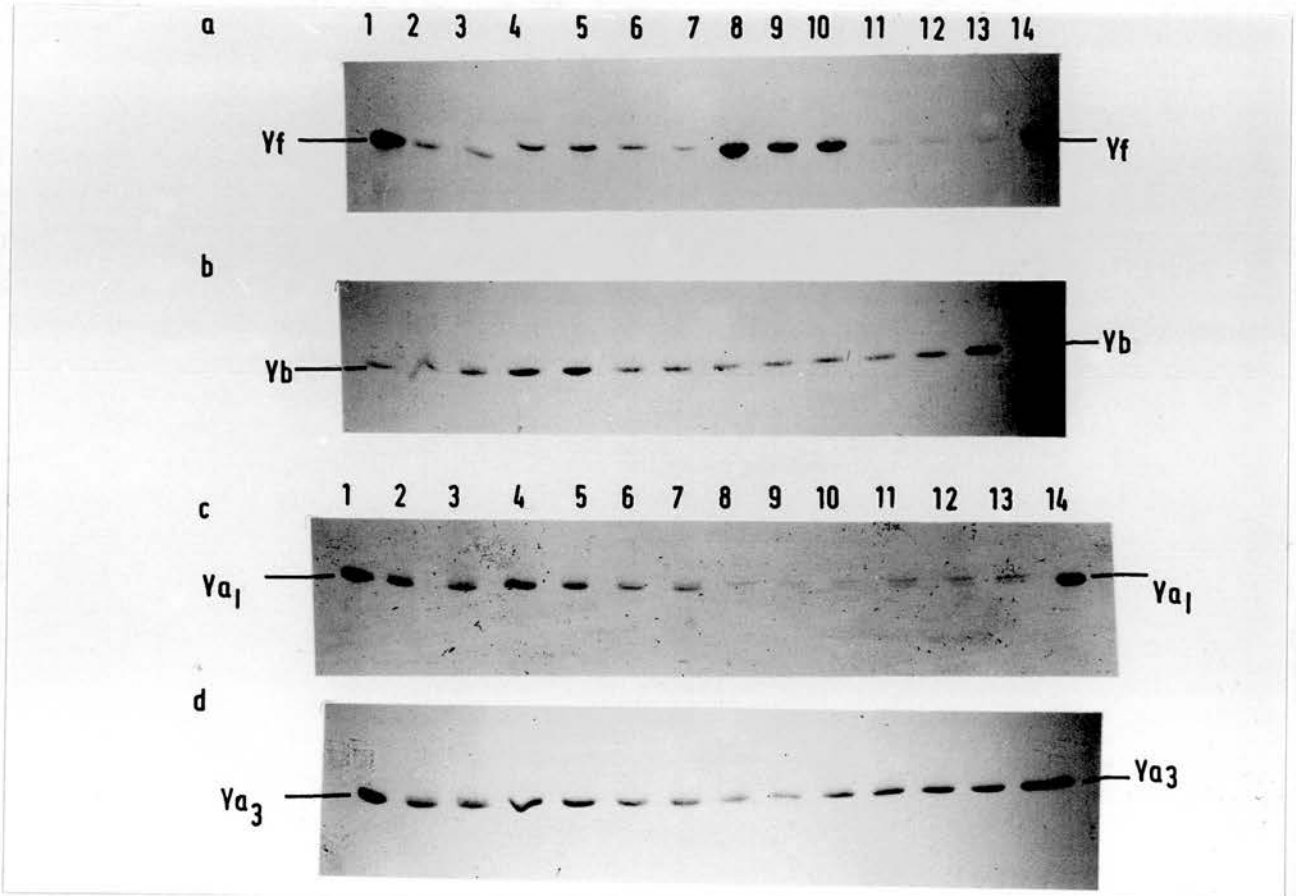
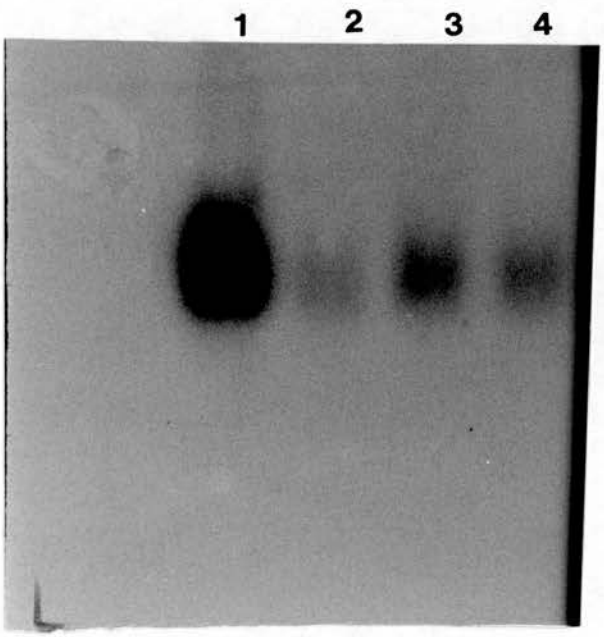

a
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Yf
Yf
b
Yb
Yb
c
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Ya1
Ya1
d
Ya3
Ya3

Figure 4.06

Northern blot analysis of hepatic mRNA levels from hypophysectomised and control C57BL/6 mice of both sexes using the rat Yf cDNA.

Hepatic RNA (15µg) was elecrophoretically size-fractionated using a 1%(w/v) agarose gel. After electrophoresis, RNA was transferred to nitrocellulose paper by capilliary action and hybribised overnight at 65°C with a P^{32}CTP-labelled Yf cDNA probe. Loadings were as follows :- lane 1, sham male operated controls; lane 2, normal female controls; lane 3, hypophysectomised males; lane 4, hypophysectomised females.



4.03 The hepatic GST complement of the little mouse.

4.03a Hepatic GST content of little mice.

An examination of the hepatic GST profile of the little mouse (lit/lit), both male and female was undertaken. Two strains of mice were used as controls throughout these experiments; a) mice heterozygous for the lit gene, (lit/+) which display normal growth characteristics and b) normal wild-type C57BL/6 mice (+/+), the progenitor strain from which the little mouse was derived.

Cytosols were prepared from pooled liver samples containing not less than 5 animals per group. The cytosol preparation was performed in duplicate. The hepatic profile from these cytosols were analysed by SDS/PAGE, followed by immunoblotting to investigate the GST profile. Little male mice were found to display a feminine pattern in respect to their GST profile, Yf levels were similar to those found in the female. Levels of the Ya_3 and Yb-type subunits did not appear to differ significantly between groups. Interestingly, the Ya_1 subunit was elevated in little mice of both sexes (figures 4.07, 4.08).

This difference was further confirmed using activity measurements for several GST substrates, table 4.05 presents the data obtained. A marked decline in CDNB and ethacrynic acid activity in the little male was observed, as would be expected. Other substrates measured remained unchanged between little mice and their controls. Messenger RNA encoding the Yf GST also showed a similar change (figure 4.09).

To further confirm this important observation, purification of hepatic GST from little mice and controls was undertaken. By this method, a quantitative estimate of GST subunit levels in little males and females could be obtained.

Figure 4.07

Electrophoretic analysis of hepatic cytosolic protein from lit/lit, lit/+ and +/+ males and females.

Duplicate cytosols preparations were undertaken from pooled liver samples (5 mice/group) from each strain and sex. Cytosolic protein (50μg/track) was analysed by SDS/PAGE on a 12% (w/v) polyacrylamide resolving gel. Loadings were as follows :- lanes 1 and 14, rat lung standards (Yc, Yb, Yf); lanes 2-3, lit/lit female; lanes 4-5, lit/lit male; lanes 6-7, lit/+ female; lanes 8-9, lit/+ male; lanes 10-11, +/+ female; lanes 12-13, +/+ male.

Figure 4.07

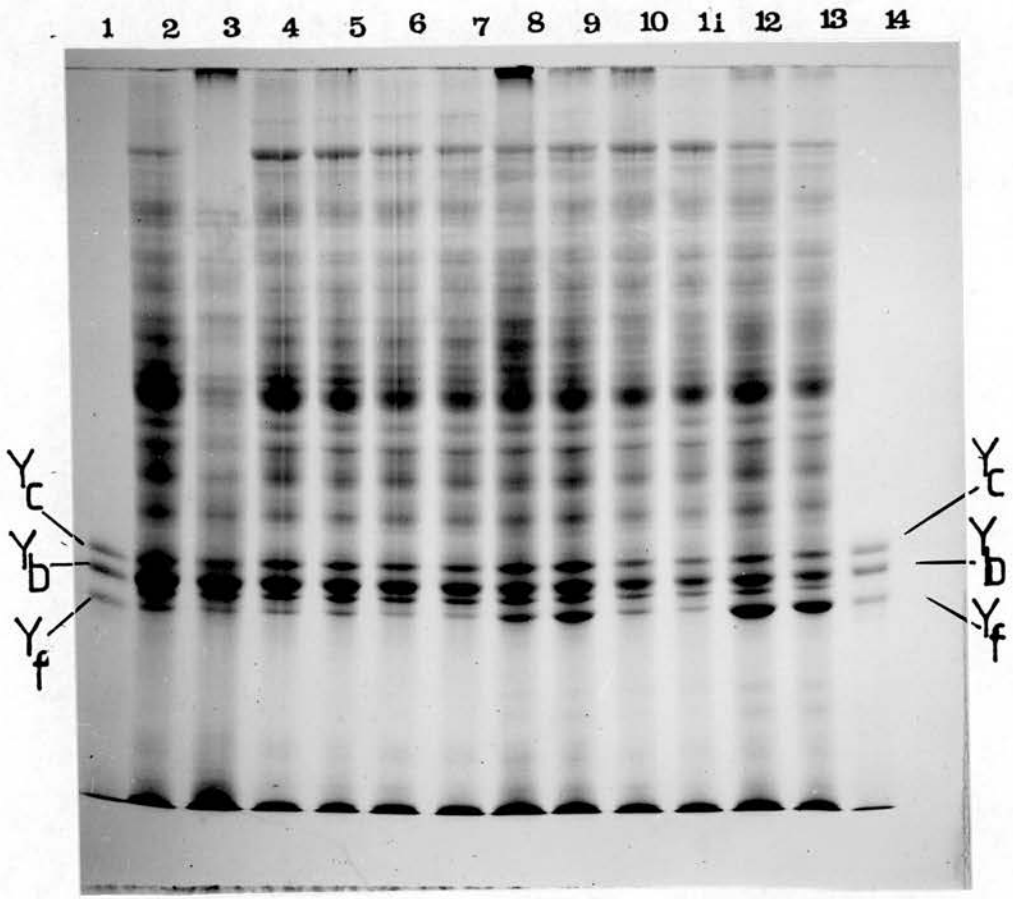


Figure 4.08

Immunoblotting of hepatic cytosols from both sexes of little mice and heterozygote and wild-type C57BL/6 controls.

Hepatic cytosols (50μg) were subject to SDS/PAGE as has been described previously (see section 2.02d) and immunoblotted against anti-mouse GST antisera.

Figure 4.08 a,d. Loadings were as follows; lanes 1and 14, purified mouse standard; lanes 2-3, lit/lit female; lanes 4-5, lit/lit male; lanes 6-7, lit/+ female; lanes 8-9, lit/+ male; lanes 10-11, +/+ female; lanes 12-13, +/+ male.

Panel a - anti-(mouse YfYf) IgG.

Panel d - anti-(mouse Yb_1Yb_1)IgG.

Figures 4.08 b,c. Loadings were as follows ; lanes 1 and 14, purified mouse GST standards; lanes 2-3, lit/lit male; lanes 4-5, lit/lit female; lanes 6-7, lit/+ male; lanes 8-9, lit/+ female; lanes 10-11, +/+ male; lanes 12-13, +/+ female.

Panel b - anti-(mouse Ya_1Ya_1) IgG.

Panel c - anti-(mouse Ya_3Ya_3) IgG.

Figure 4.08

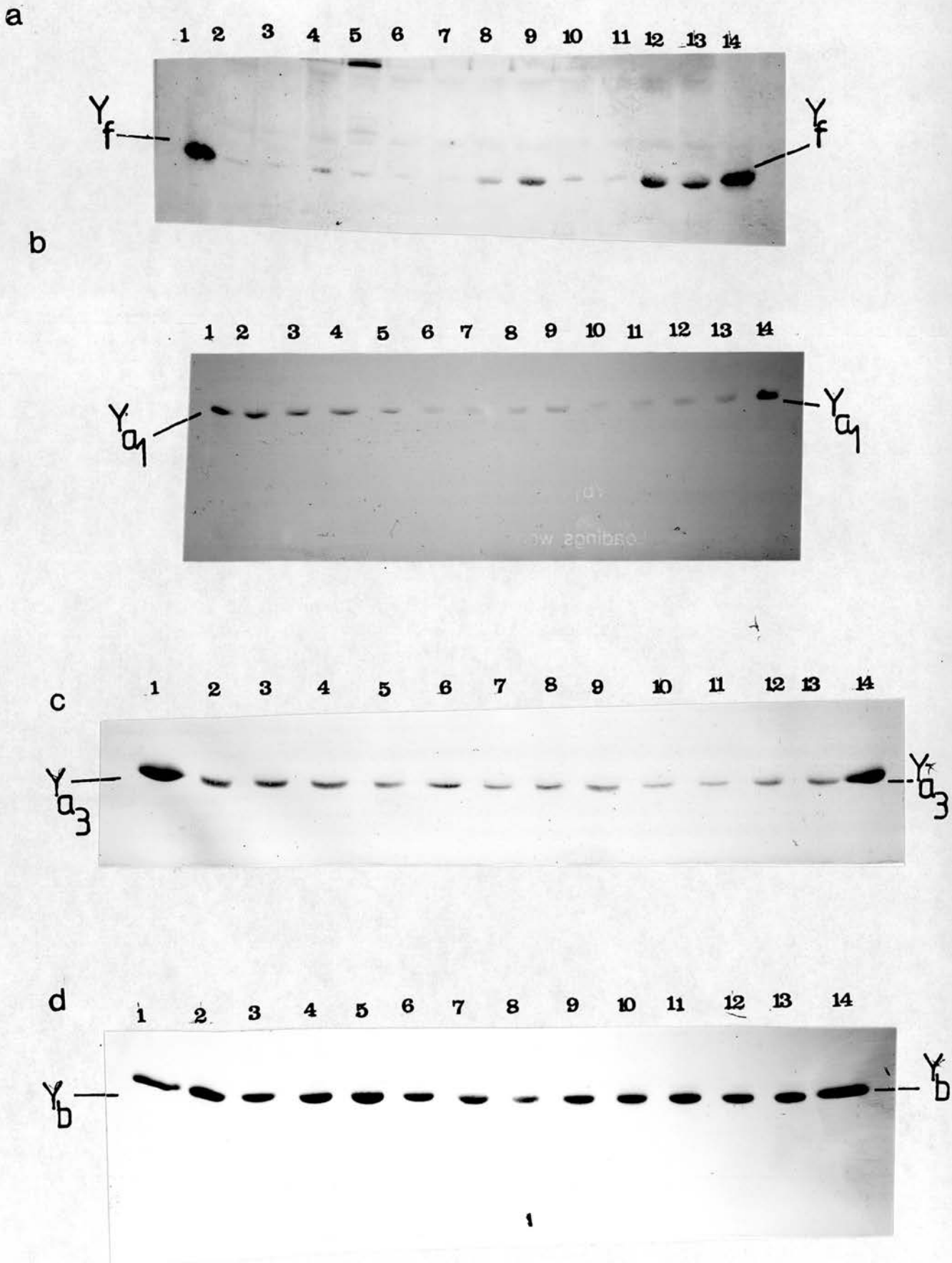

a
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Yf
Yf
b
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Ya1
Ya1
c
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Ya3
Ya3
d
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Yb
Yb

Table 4.05 Levels of GST actvity in hepatic cytosols from little mice and heterozygote and wild-type controls

Strain	Sex	CDNB	DCNB	Ethacrynic Acid	CuCOH	H_2O_2
Lit/Lit	Male	5.72**	0.137	0.047*	1.97	1.17*
		(0.59)	(0.02)	(0.01)	(0.13)	(0.06)
	Female	5.72**	0.128	0.043**	1.75	1.17
		(0.72)	(0.02)	(0.01)	(0.14)	(0.1)
Lit/+	Male	9.1	0.148	0.12	1.76	1.33
		(0.74)	(0.03)	(0.00)	(0.13)	(0.13)
	Female	5.3*	0.127	0.039*	2.21	1.68
		(0.55)	(0.02)	(0.002)	(0.1)	(0.08)
+/+	Male	9.8	0.153	0.092	1.87	1.30
		(1.08)	(0.03)	(0.02)	(0.01)	(0.02)
	Female	4.3*	0.94	0.023*	1.72	1.4
		(0.17)	(0.003)	(0.012)	(0.1)	(0.8)

Standard deviations are presented in bracketts.

Mean values for each group are quoted. * P > 0.01, ** P > 0.001

Degrees of significance are assessed relative to the +/+ male control Units - µmoles/min/mg protein

All assays were performed in triplicate on cytosols prepared from individual mice,

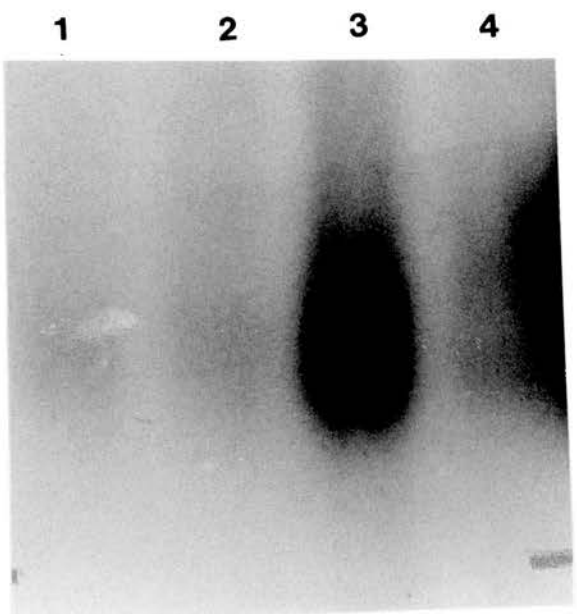
number of mice per group - male and female little mice groups, n=6

-male and female wild-type and heterozygote groups, n=3

Figure 4.09

Northern blot analysis of little and heterozygote hepatic mRNA levels using the rat Yf cDNA.

Hepatic Yf mRNA levels were measured by Northern blot analysis as described in section 2.12. Liver RNA (20μg) was electrophorectically size-fractionated using a 1% (w/v) agarose gel with 2.2M-formaldehyde. After electrophoresis the RNA was transferred to nitrocellulose and hydridised overnight at 65°C with the rat Yf cDNA probe labelled with P^{32}CTP. Loadings were as follows; lane1, lit/lit male; lane 2, lit/lit female; lane3, lit/+ male; lane 4, lit/+ female.



4.03b Purification of the hepatic GST mixture from little mice and their controls

Purification of GST from little males and little females (lit/lit), from male and female heterozygote (lit/+) as well as from wild-type controls (+/+) was undertaken. GST isoenzyme mixtures were isolated from mouse livers using glutathione affinity chromatography as described in section 2.05a.

Recovery of GST from the GSH-affinity column ranged from 74% to 94 %. The level of GST expressed as a percentage of total cytosolic protein was found to be slightly higher in the wild-type and heterozygote males as compared to females and little males (table 4.06).

Table 4.06 Recovery of Hepatic GST after GSH-Affinity Chromatography

Strain	Sex	Level of GST as a % total cytosolic protein	% CDNB activity retained after GSH-affinity chromatography
Lit/Lit	male	2.0	87
	female	3.0	94
Lit/+	male	4.0	92
	female	2.0	91
+/+	male	3.5	74
	female	1.6	89

Figure 4.10

SDS/PAGE of purified hepatic isoenzyme mixtures from lit/lit, lit/+ and +/+ male and females.

Mouse GST from liver cytosols of each strain and sex were purified by GSH-affinity chromatography and the subunit content analysed by SDS/PAGE on a 12% (w/v) polyacrylamide resolving gel. The gel was loaded with 3μg of GST in each lane, loadings were as follows :- lanes 1 and 8, rat liver markers, (Yc,Yb, Ya); lane 2, lit/lit male; lane 3, lit/lit female; lane 4, lit/+ male; lane 5, lit/+ female; lane 6, +/+ male; lane 7, +/+ female.

Figure 4.11

Immunoblotting of purified hepatic GST isoenzyme mixtures from little males and females and heterozygote and wild-type controls of both sexes.

Hepatic mouse GST (3μg protein/track) from each strain and sex were purified by affinity chromatography, subjected to SDS/PAGE, transferred to nitrocellulose and analysed by immunoblotting with anti-(mouse YfYf) IgG. The gel was loaded as follows:- lane1 and 8, mouse Yf markers; lane2, lit/lit male; lane 3, lit/lit female; lane4, lit/+ male; lane 5, lit/+ female; lane 6, +/+ male; lane 7, +/+ female.

Figure 4.10

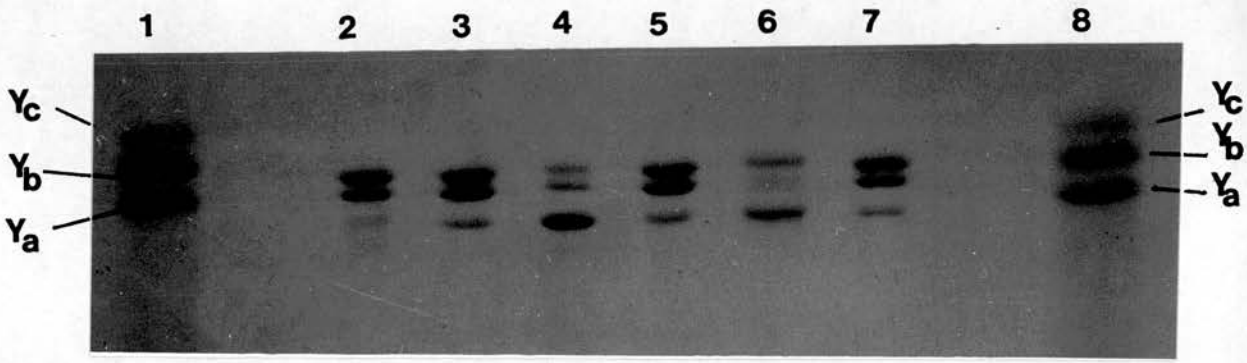
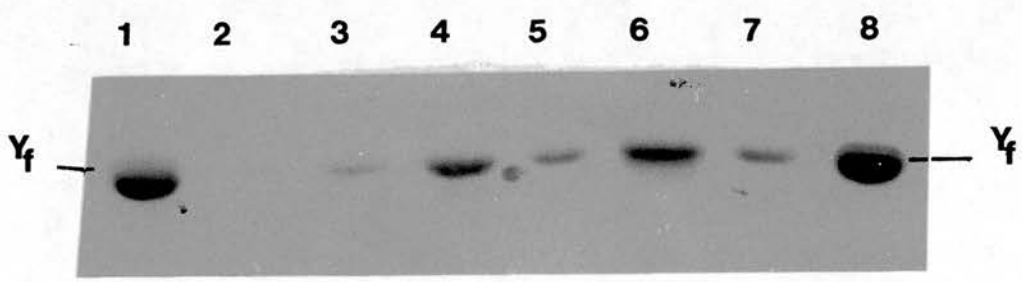


Figure 4.11



Hydroxyapatite h.p.l.c. analysis of GSH-affinity purified GST pools

Individual GST subunits were resolved from the GSH affinity purified pools by hydroxyapatite h.p.l.c. as described in section 2.05b. The h.p.l.c. traces obtained from little, heterozygote and wild-type males and females are presented in figures 4.12, 4.13, 4.14 respectively.

In all groups and sexes, three distinct peaks were obtained with retention times of 36min, 41min, 50min. From previous work from this laboratory, it is known that the Yf GST elutes first, followed by Ya class subunits and finally Yb-type subunits (Hayes *et al.*, 1987; McLellan and Hayes, 1897). Little males displayed a similar trace to that obtained for female mice. Fractions were collected at 1min intervals and assayed for protein and CDNB activity. Fractions from each peak containing the highest CDNB activity were pooled. The peak of A_{280}-absorbing material eluting at 13min did not contain protein and did not possess any activity towards CDNB; it is most probably GSH.

Due to the scarcity of starting material, an exhaustive characterisation of purified peak fractions could not be undertaken however specific activities towards CDNB were obtained for the purified pooled fractions. These are given in table 4.07. Values quoted are of a similar order to those determined by McLellan and Hayes (1987). Peak no.3, containing Yb type subunits, had the highest activity towards CDNB, followed by peak no.1 (Yf), the Ya (peak no.2) had the lowest specific activity.

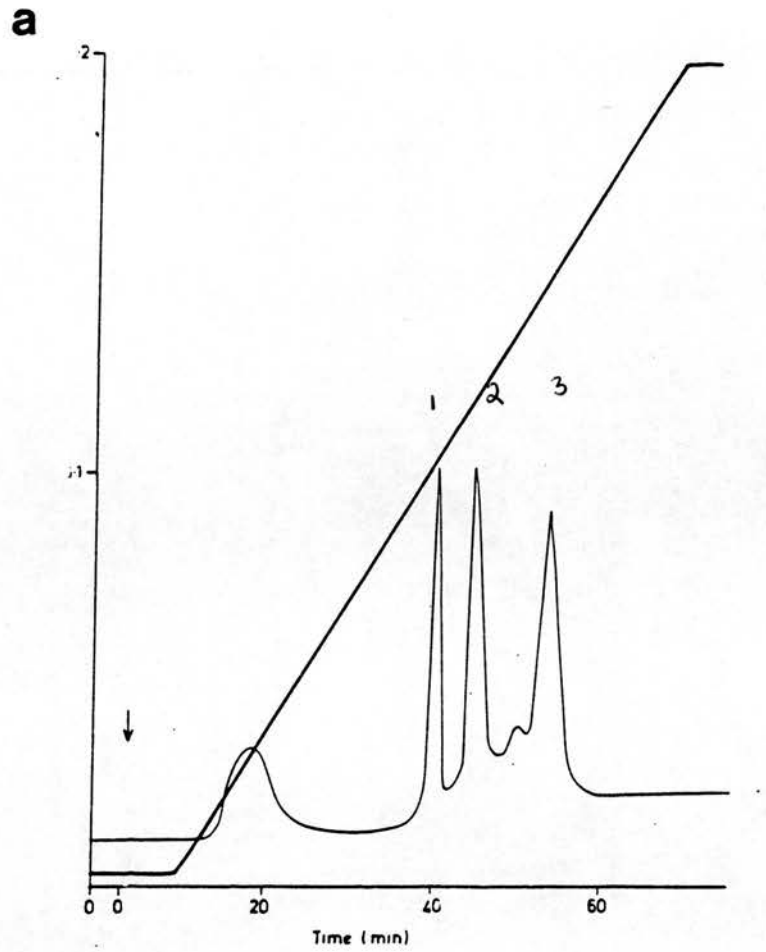
Table 4.08 gives the relative yields obtained for individual subunits from h.p.l.c. analysis. In control males, the Yf GST subunit was found to constitute approximately 70% of total GST, by far the major GST in normal male liver. In contrast, the Yb was

Figure 4.12 Use of hydroxyapatite h.p.l.c. to resolve mouse liver GST subunits from male and female little mice.

Affinity purified pools were subject to chromatography on Bio-gel HPHT. Portions (2ml, 0.26mg of protein for both sexes) were applied to hydroxyapatite and were eluted from the h.p.l.c. coloumn at 0.5ml/min with a liear gradient of 10-350 mM-sodium phosphate which contained 0.5mM-DTT. Protein peaks were detected by monitoring the absorbance at 280nm. The GST peaks were eluted by monitoring the absorbance at 280nm. The GST peaks were eluted at 36min, 41min and 50min. Panels (a) and (b) show elution profiles of hepatic GST from male and female little mice.

Peak 1 = 36min
Peak 2 = 41min
Peak 3 = 50min

Figure 4.12


a
1
2
3
0 0
20
40
60
Time (min)

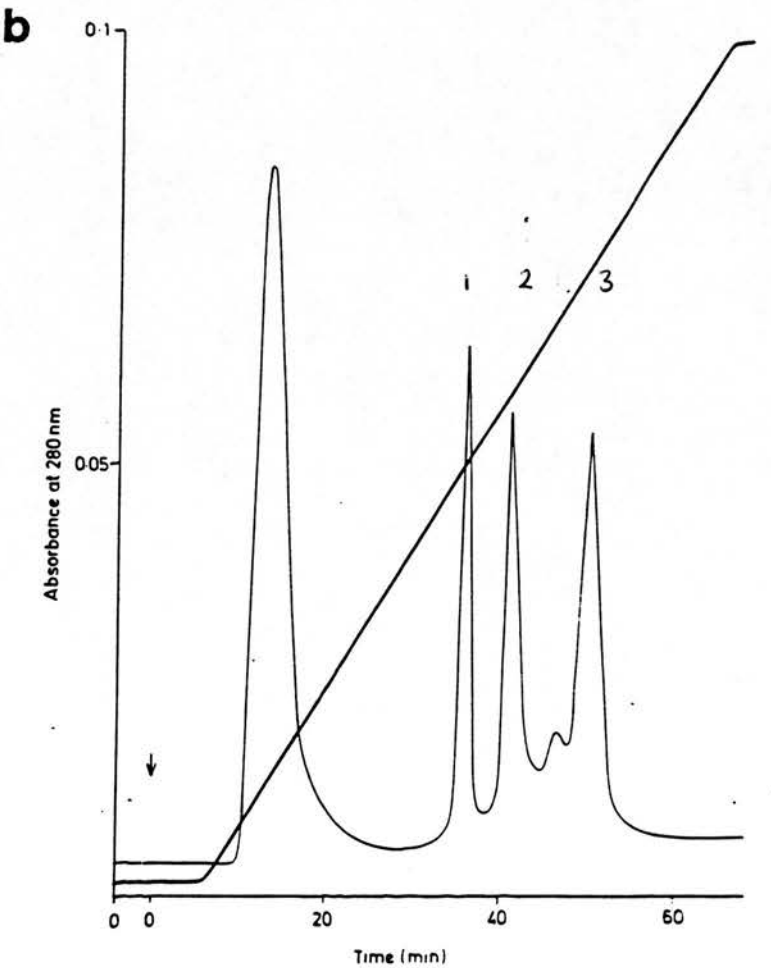

b
0·1
0·05
Absorbance at 280 nm
1
2
3
0 0
20
40
60
Time (min)

Figure 4.13 Use of hydroxyapatite h.p.l.c. to resolve mouse liver GST from male and female heterozygotes (lit/+).

Affinity purified GST (2ml, 0.98mg and 0.31mg of protein for male and female respectively) were subject to chromatography on Bio-Gel HPHT as described in the legend to figure 4.12. GST peaks eluted at 36min, 41min and 50min. Panel (a) shows the elution profile for hepatic GST from male heterozygotes. Panel (b) shows the hepatic GST elution profile for female heterozygotes.

Peak 1 = 36min
Peak 2 = 41min
Peak 3 = 50min

Figure 4.13

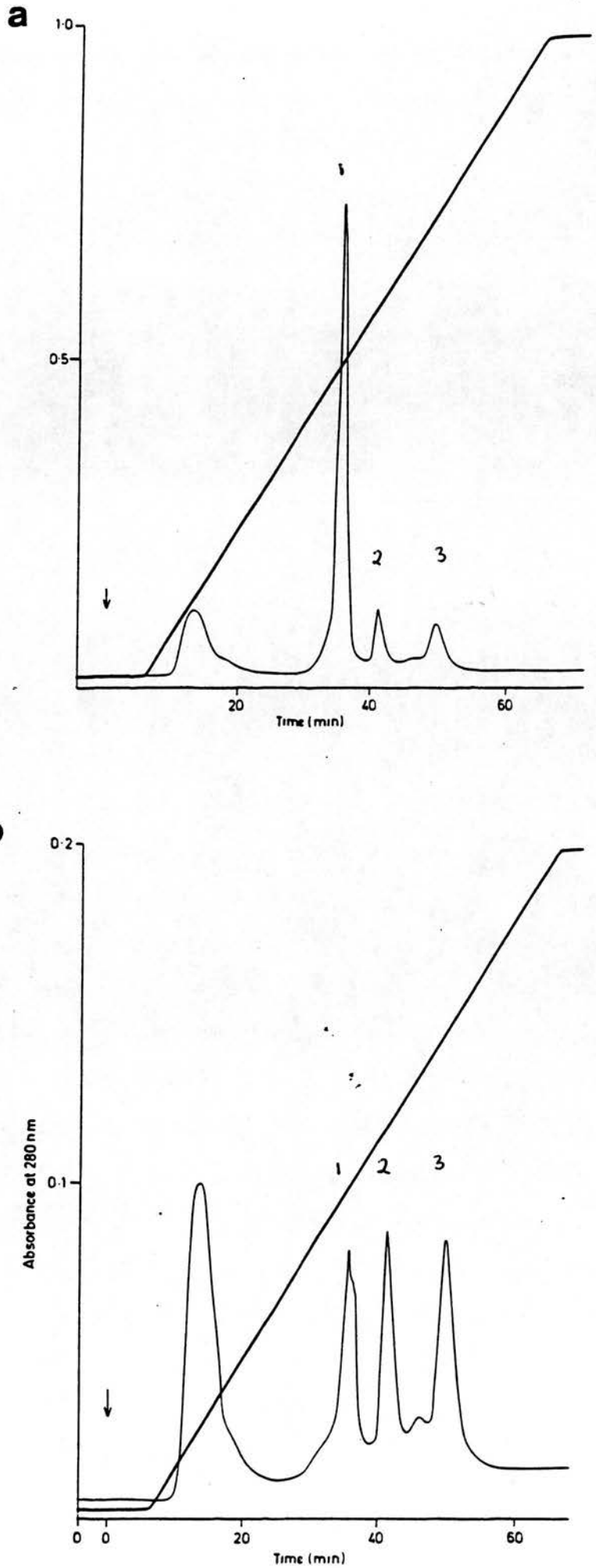

a
1·0
0·5
1
2
3
20
40
60
Time (min)
b
0·2
0·1
Absorbance at 280 nm
1
2
3
0
0
20
40
60
Time (min)

Figure 4.14 Use of hydroxyapatite h.p.l.c. to resolve mouse liver GST from wild-type (+/+) males and females of the C57BL/6 strain.

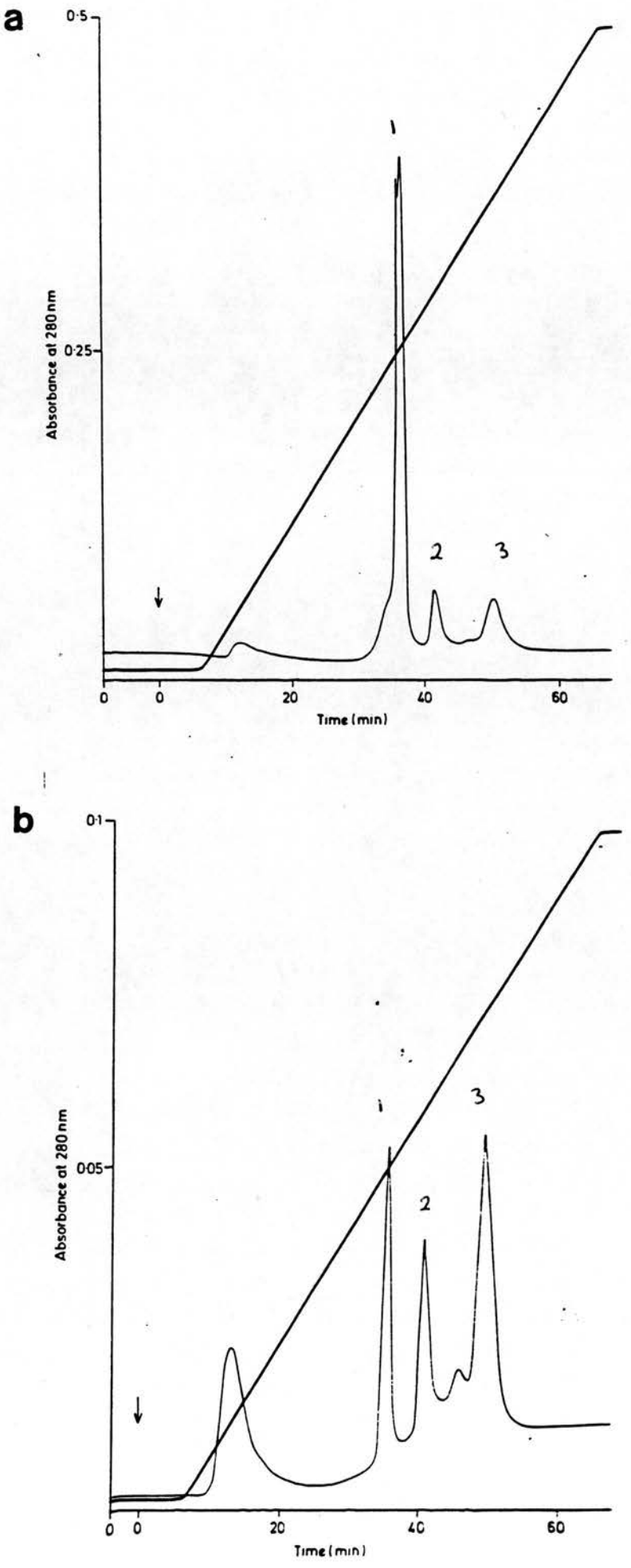
Affinity purified GST (2ml, 0.92mg and 0.25mg of protein for male and female respectively) were subject to chromatography on Bio-Gel HPHT as described in the legend to figure 4.12. GST peaks eluted at 36min, 41min and 50min. Panels (a) and (b) show the elution profiles for male and female C57BL/6 mice.

Peak 1 = 36min

Peak 2 = 41min

Peak 3 = 50min

Figure 4.14


a
0·5
0·25
Absorbance at 280 nm
1
2
3
0
0
20
40
60
Time (min)
b
0·1
0·05
Absorbance at 280 nm
1
2
3
0
0
20
40
60
Time (min)

the major GST little males, with the Yf forming only 23% of the total GST content. A similar situation to that observed in females.

Table 4.07 Specific Activities towards CDNB of purified GST subunits

(units of activity - µmoles/min/mg protein)

Group	Sex	Peak1 (36min) (Yf)	Peak2 (41min) (Ya)	Peak3 (50min). (Yb)
Lit/Lit	Male	81.3	20.0	141.3
	Female	93.1	18.2	149.0
Lit/+	Male	112.2	28.4	139.0
	Female	114.0	17.0	143.6
+/+	Male	93.4	26.9	129.0
	Female	105.5	20.6	149.1

4.03c Plasma Testosterone Levels in the Little Male.

Plasma testosterone levels in the little mouse were measured by the method of Corker *et al.* (1978). The results are presented in table 4.09 The testosterone concentration in the little male were found to be very similar to that found in the heterozygote and wild-type males. In females, testosterone levels are extremely low, 0.6nmol/l or less, which are beyond the lower limits of detectabilty in this assay.

Table 4.08 Recovery of GST subunits from h.p.l.c.

Group		Yields of GST subunit types from h.p.l.c. as a percentage of total protein recovered			Levels of Yf expressed as a % of Ya +Yb	Levels of Ya expressed as a percentage of Ya + Yb
		Yf	Ya	Yb		
Lit/Lit	Male	23.2	31.6	45.2	30.2	41.2
	Female	27.4	27.6	45.0	37.8	38.0
Lit/+	Male	76.5	10.9	12.6	326	46.3
	Female	31.8	27.9	40.3	46.6	41.0
+/+	Male	77.0	10.3	12.6	336	44.9
	Female	23.9	26.2	49.8	31.4	34.5

Table 4.09 Testosterone levels in little male mice and heterozygote and wild-type male controls.

Strain	Testosterone Concentration (nmol/l)	
Lit/Lit	3.8	(n=9, sd=3.5)
Lit/+	4.0	(n=8, sd=2.8)
+/+	3.8	(n=6, sd=4.1)

Abbreviations: n, number; sd, standard deviation.

4.04 Treatment of Little Mice with Growth Hormone and Testosterone.

Little mice and their heterozygote and wild-type controls were obtained from Charles Rivers Laboratories, Willmington, Massachusetts. After transit, the mice were acclimatized at Clair Hall laboratories, Oxford for 2 weeks prior to the commencement of treatment. Little mice received either testosterone propianote, subcutaneously injected in corn oil at 120μg/mouse or recombinant human growth hormone (Genotropin), injected subcutaneously in sterile water in twice daily doses of 5μg (0.03 international units). These treatments were administered for 7 consecutive days, the injections being separated by approximately 12 hours to simulate the male pattern of growth hormone secretion. Control animals received injection vehicle only. Heterozygote and wild-type controls were subjected to

replacement therapy with growth hormone only. A summary of treatment groups and group numbers is given in table 4.10.

Cytosols, prepared from individual mice, were measured for activity towards CDNB. No change in CDNB activity was noted in any of the groups after treatment with either hormone (figure 4.15). Following SDS/PAGE however, a protein of Mr 24,800 was found to have been induced in the little males on treatment with growth hormone. No effect was observed after testosterone administration (figure 4.16). A similar pattern of events was demonstrated in the little female (figure 4.17). Hormone therapy had no appreciable effect on wild-type males and females. Immunoblotting with anti-mouse YfYf antisera gave a similar pattern of events (figure 4,18, 4.19). Results obtained for the heterozygotes mirrored those observed for wild-type controls (data not shown).

4.05 Discussion

4.05a Summary of results.

The aim of the work described in this chapter was to examine the role of the pituitary gland in the regulation of hepatic GST expression in the mouse. The evidence presented indicates that the sex-specific regulation of the Yf subunit is controlled via the pituitary gland, more specifically by growth hormone which is produced by the pituitary somatotrophs.

Hypophysectomy of male C57BL/6 mice results in a sharp decline in the levels of the Yf subunit. In the female, a slight elevation in hepatic Yf was noted after removal of the pituitary.

Table 4.10 Treatment groups for little mice hormone replacement therapy

Strain	Sex	Treatment	Group number
Lit/Lit	Male	water	3
		GH	3
		corn oil	3
		testosterone	3
Lit/Lit	Female	water	3
		GH	3
		corn oil	3
		testosterone	3
Lit/+	Male	water	4
		GH	3
	Female	water	3
		GH	3
+/+	Male	water	3
		GH	3
	Female	water	3
		GH	3

Abbreviations: GH - growth hormone

Figure 4.15

Levels of CDNB activity in hepatic cytosols from little mice undergoing hormone replacement therapy.

a. little mice of both sexes treated with growth hormone (GH).

b. little mice of both sexes treated with testosterone (test).

c. heterozygote mice treated of both sexes treated with GH.

d. wild-type mice of both sexes treated with GH.

con, control.

Figure 4.15

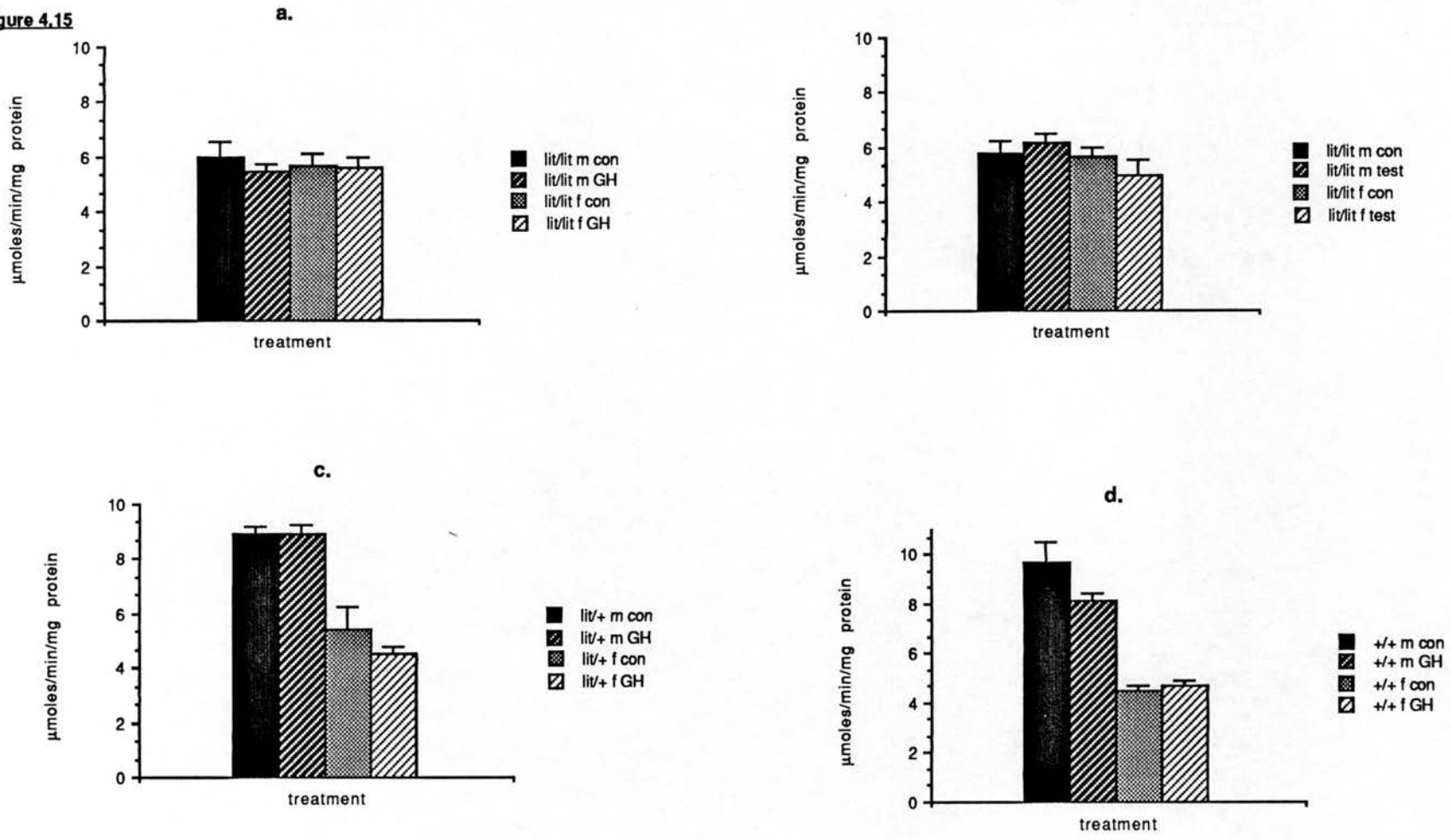

a.
µmoles/min/mg protein
10
8
6
4
2
0
treatment
lit/lit m con
lit/lit m GH
lit/lit f con
lit/lit f GH
b.
µmoles/min/mg protein
10
8
6
4
2
0
treatment
lit/lit m con
lit/lit m test
lit/lit f con
lit/lit f test
c.
µmoles/min/mg protein
10
8
6
4
2
0
treatment
lit/+ m con
lit/+ m GH
lit/+ f con
lit/+ f GH
d.
µmoles/min/mg protein
10
8
6
4
2
0
treatment
+/+ m con
+/+ m GH
+/+ f con
+/+ f GH

Figure 4.16

Electrophoretic analysis of hepatic cytosols from little male mice undergoing hormone replacement therapy.

Hepatic cytosols (30μg/track) from little male mice undergoing hormone replacement therapy were analysed by SDS/PAGE using a 12% (w/v) polyacrylamide resolving gel. The gel was loaded as follows :- lane 1, rat liver markers, (Yc, Yb, Ya); lanes 2-4, lit/lit male controls; lanes 5-7, lit/lit males treated with growth hormone; lanes 8-10, lit/lit male controls; lanes 11-13, lit/lit males treated with testosterone; lanes 14-16, +/+ male controls; lanes 17-19, +/+ males treated with growth hormone, lane 20, purified mouse Yf standard.

Figure 4.17

Electrophoretic analysis of hepatic cytosols from little female mice undergoing hormone replacement therapy.

Hepatic cytosols (30μg/track) from lit/lit females were analysed by SDS/PAGE using a 12% (w/v) polyacrylamide resolving gel. Loadings were as follows :- lane 1, rat liver markers (Yc, Yb, Ya), lanes 2-4, lit/lit female controls; lanes 5-7, lit/lit females treated with growth hormone; lanes 8-10, lit/lit females controls; lanes 11-13, lit/lit females treated with testosterone; lanes 14-16, +/+ controls, lanes 17-19, +/+ females treated with growth hormone; lane 20, purified mouse Yf standard.

Figure 4.16

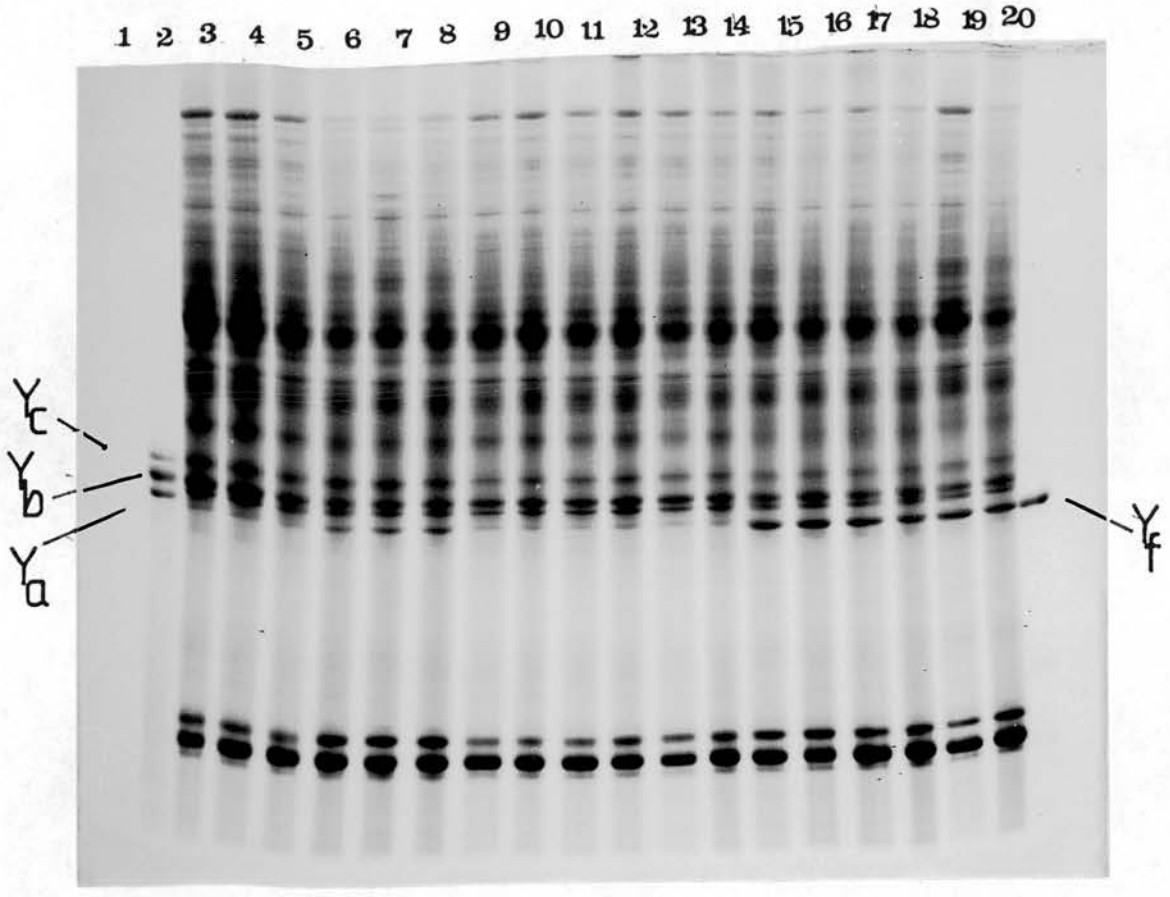


Figure 4.17

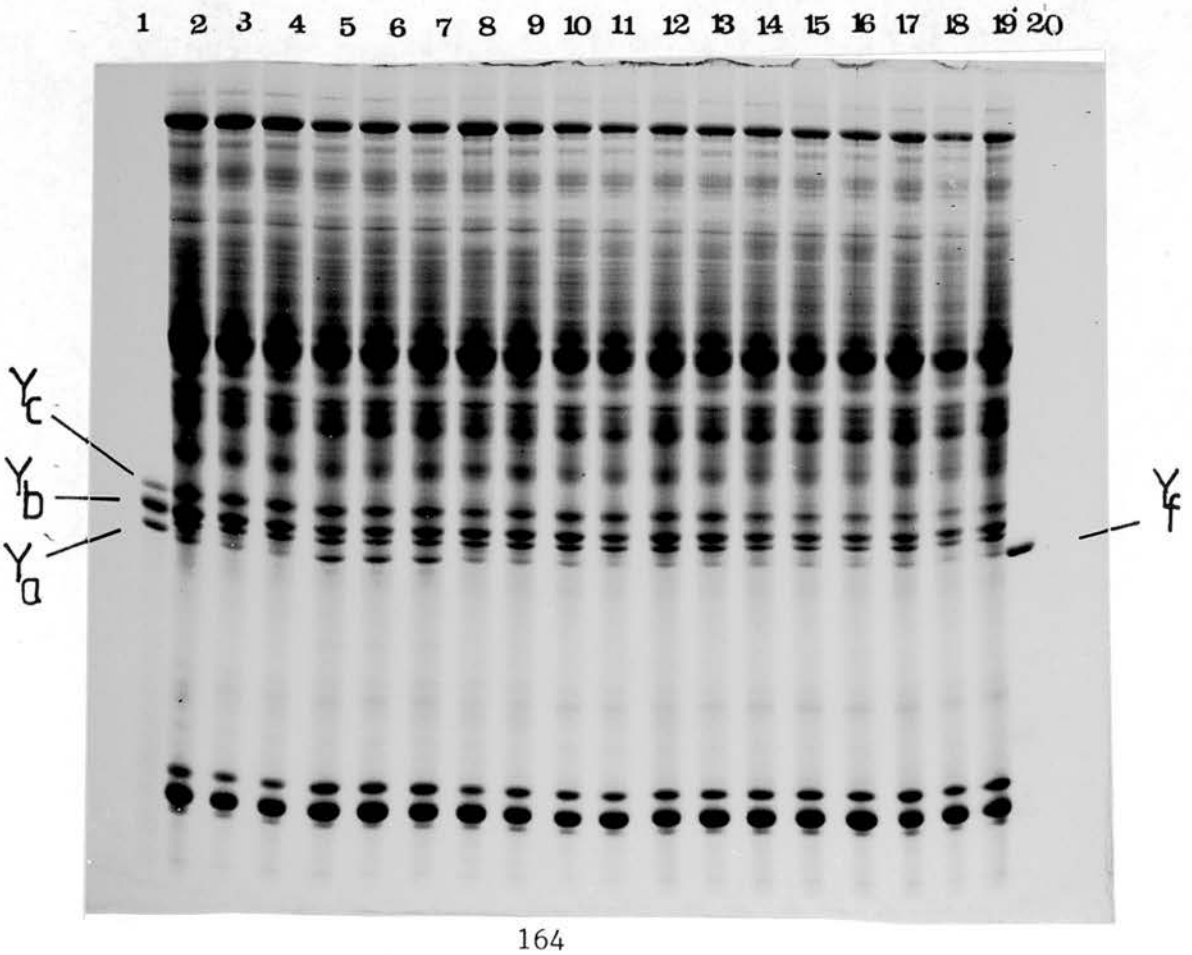


Figure 4.18

Immunoblotting of hepatic cytosols from little males undergoing hormone replacement therapy.

Hepatic cytosols (50μg protein/track) from little males undergoing hormone replacemant therapy were subject to SDS/PAGE followed by transfer to nitrocelluose and immunoblotting with anti-(mouse YfYf) IgG. gel loadings were as follows :- lanes 1 and 17, mouse Yf standard; lanes 2-4, lit/lit male controls; lanes 5-7, lit/lit males treated with growth hormone; lanes 8-10, lit/lit males treated with testosterone; lanes 11-13, +/+ male controls; lanes 14-16, +/+ males treated with growth hormone.

Figure 4.19

Immunoblotting of hepatic cytosols from little females undergoing hormone replacement therapy.

Hepatic cytosols (50μg protein/track) from little females undergoing hormone treatment were subject to SDS/PAGE, transferred to nitrocellulose and immunoblotted with anti-(mouse YfYf)IgG. Gel loadings were as follows :- lanes 1 and 17, mouse Yf standard; lanes 2-4, lit/lit female controls; lanes 5-7, lit/lit females treated with growth hormone; lanes 8-10, lit/lit females treated with testosterone; lanes 11-13, +/+ female controls; lanes 14-16, +/+ females trated with growth hormone.

Figure 4.18

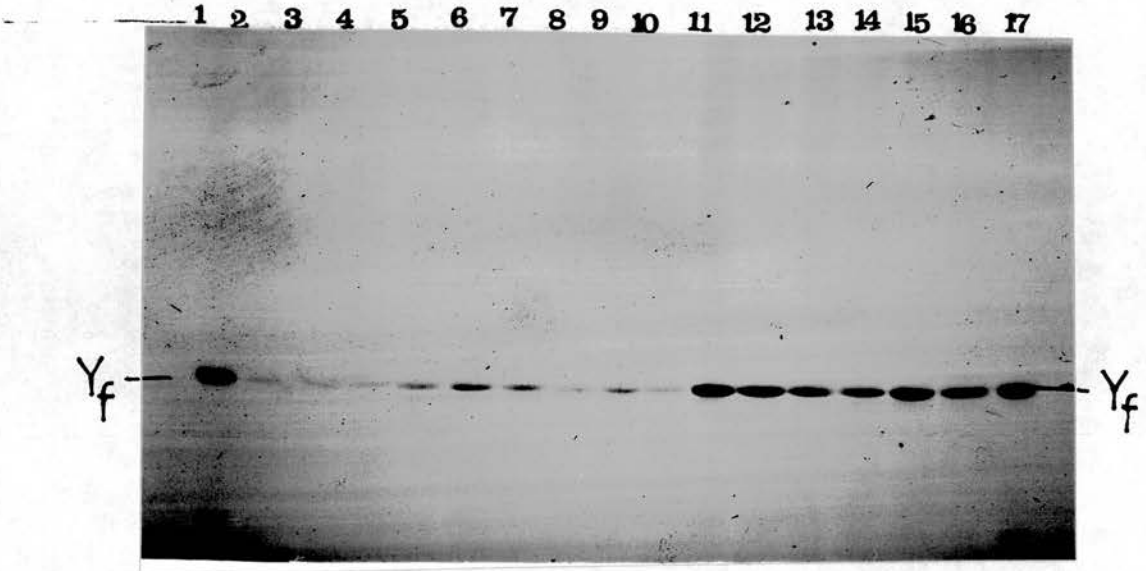
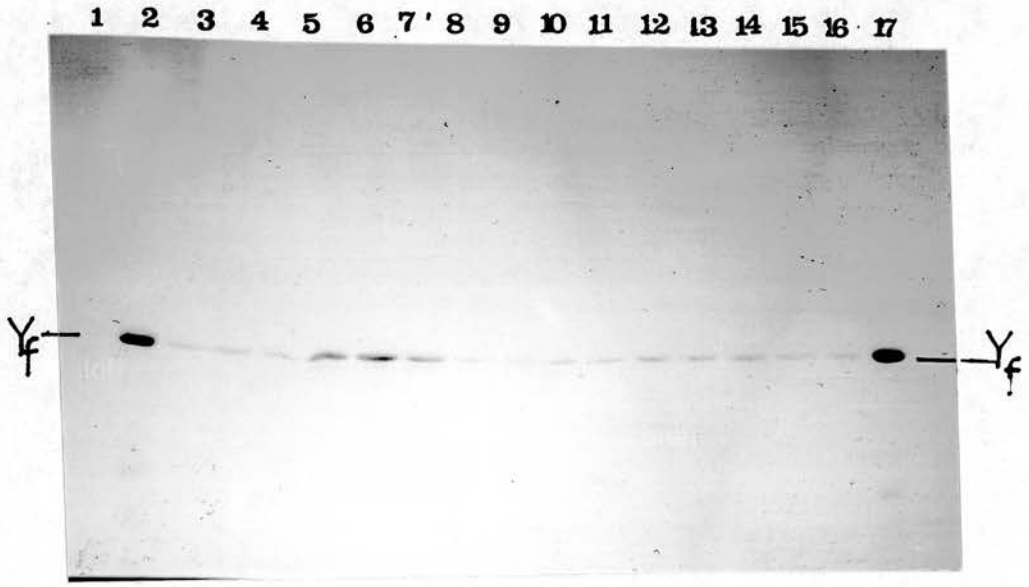


Figure 4.19



Characterisation of the hepatic GST profile in the little mouse demonstrated that the absence of growth hormone led to a feminine phenotype in the male with respect to the Yf polypeptide. Northern blot analysis showed that this difference in Yf content occured at the pretranslational level, although it is not known whether this is due to an decrease in the transcription of the Yf gene or its stability.

Circulating testosterone levels have not been reported for the little mouse. Fertility problems have been noted in this mutant strain (Beamer and Eicher, 1976). To exclude the possibility that low testosterone levels in the little mouse might account for the finding detailed above, plasma was collected from these mice and testosterone levels measured. Plasma testosterone concentrations were found to be similar to the heterozygote and wild-type strains. Moreover the values obtained are in good agreement with previously quoted levels of testosterone in the C56BL/6 strain (Batty, 1978; Kemp and Drinkwater, 1989). Steroidogenesis in the little mouse has also been found to be normal as have testicular testosterone levels (Chubb and Nolan, 1985; Chubb, 1987). This would indicate that the lack of testosterone is not the factor responsible for the low levels of YfYf in the little male.

A marked sexual dimorphism in the expression of P-450 isozymes in mouse kidney has been noted. Unlike the hepatic P-450 enzymes described in section 4.01a which are not controlled directly by testosterone, androgens appear to mediate the sexual differentiation of kidney P-450 enzymes directly. In little males and females, the P-450 profiles are normal, suggesting again that testosterone levels are not depressed (Henderson *et al.*, 1990).

In the little mouse, testosterone levels are normal and yet in the male a feminine pattern of GST expression is observed. Moreover, treatment of little mice with testosterone, at concentrations which have been shown to induce Yf in intact females and castrated C57BL/6 males, does not restore the expression of Yf to levels observed in wild-type males. It should be emphasised from the work of Henderson *et al* (1990) that enzymes in both castrated and little mice which are directly controlled via testosterone respond in a normal manner to treatment with this hormone. These data indicated strongly that testosterone is not regulating Yf levels through a direct mechanism.

When, however, little mice were subject to replacement therapy with growth hormone, an elevation in the expression of the Yf subunit was observed in both the male and female. By contrast, no change was observed in GST expression in controls treated with growth hormone. This evidence strongly suggests that that growth hormone deficiency in the little mouse leads to female-type expression of the Yf subunit, correcting this deficiency by growth hormone administration leads to an induction of hepatic Yf, although this treatment by no means restores expression of Yf to normal male levels.

Although growth hormone treatment induced the Yf in little mice, the level of expression was considerably lower than that found in heterozygote and wild-type male controls. The low level of induction could possibly be due to the treatment regime employed. MacLeod and Shapiro (1989) demonstrated that a daily dose of rat growth hormone at a concentration of 0.08 i.u. / 100g body weight divided into 2 subcutaneous injections for a duration of 7 days could masculinise drug metabolism in hypophysectomised and/ or gonadectomised male mice. A similar concentration of recombinant human growth hormone was used in this study. It is possible that

growth hormone from another rodent species would be more physiologically active in the mouse. Other workers have shown human recombinant growth hormone to be active in the mouse (Norstedt and Palmiter,1984); unfortunately, the international units used by these workers was not quoted. It is therefore difficult to estimate the dose administered for comparison to the present work. Human growth hormone has certainly been used in the rat but at a dose 4 x higher than than has been used in this experiment(Waxman *et al.*, 1988).The duration of treatment in most cases was for 7 consecutive days. Probably a higher dose for a longer duration would have shown a greater effect.

Not only is the dose of growth hormone administered and the duration of treatment important in any observed effect but also the frequency of injection. Jansson and his co-workers looked at the longitudinal bone growth in hypophysectomised animals receiving growth hormone in subcutaneous injections at different frequencies. A daily replacement dose of bovine growth hormone was divided into 1,2,4 and 8 daily injections. A greater response was observed when growth hormone was administered as 4 daily injections, presumably this mimicked most closely the normal male secretory pattern (Jansson *et al.*, 1982). For experimental convenience, most workers employ a twice daily administration of hormone, 12 hours apart.

4.05b Is the Yf GST regulated by the growth hormone secretion?

In this study, growth hormone was administered as intermittent injections at approximately 12 hour intervals throughout a 7 day period. This mimicked the male rhythm of release from the pituitary. Induction of the Yf subunit was noted in the little mice of both sexes. The converse experiment, involving the chronic

administration of growth hormone via a mini-osmotic pump, to simulate the female growth hormone pattern, was not performed due to difficulties in obtaining both mice and growth hormone. It is not possible therefore to state conclusively that expression of the Yf subunit is regulated via the male secretory growth hormone pattern but there are several indications that this is probably the case.

From a simple analysis of the little mouse liver GST complement, growth hormone appears to be involved in the regulation of the Yf subunit. In the male little mouse (which possesses a block in growth hormone synthesis), a feminine pattern of GST expression is observed. When the growth hormone deficiency in little mice was replaced in the male fashion an increase in the expression of Yf was noted in both the male as well as the female.

The ability to induce Yf in female little mice by growth hormone treatment is perhaps surprising, but it seems likely that it is the discontinuous manner in which this hormone was administered that is responsible for the overexpression of the Yf GST. The fact that treatment of female C57BL/6 mice with testosterone causes an induction in levels of Yf appears to contradict the hypothesis that testosterone does not act directly in the of control Yf expression. However, testosterone treatment has been shown to masculinise the growth hormone secretory pattern. Removal of the testes has been found to feminise the male pattern of growth hormone secretion (Jansson *et al.*, 1985; Jansson and Frohman, 1987). Gonadectomy of the male is also associated with a decline in the expression of the Yf (see chapter 3). Interestingly, male rats exhibiting the tfm defect are found to have a growth hormone secretory pattern similar to that found in neonatally gonadectomised male rats (Millard *et al.*, 1982). No studies on growth hormone release in the tfm or hpg mouse strains have

been reported in the literature but the prediction would be that the males of these mutant mouse strains would exhibit a feminine pattern of growth hormone release.

4.05c Mechanism of action

The mechanism whereby the sexually differentiated rhythm of growth hormone secretion leads to a dimorphic expression of hepatic proteins between male and female is one of the key questions in this area, which, as yet, remains unanswered. It is not known if this effect is regulated directly by growth hormone binding to its receptor on the surface of the hepatocyte. Alternatively, growth hormone regulation might occur indirectly via the somatomedins. These are a family of hormones synthesized and released from the liver in response to growth hormone binding to its receptor. Close structural and functional similarities have been noted between the somatomedins and insulin. In humans, three somatomedins are produced, A, B, and C, and these hormones are also referred to as insulin-like growth factors (IGF). Somatomedin C is identical to insulin-like growth factor I (IGF I). After release from the hepatocyte these hormones bind to specific receptors on the surface of a number of different cell-types, including the liver. Mitogenic responses to growth hormone occur through the sommatomedins (for review, see Zapf *et al.*, 1981).

Norstedt and Palmiter (1984) have proposed a mechanism based on this dichotomy of growth hormone action. They propose that proteins induced when growth hormone is secreted in a femimine pattern, e.g. prolactin receptors, are directly regulated by growth hormone binding to its receptor. In contrast, growth hormone would regulate male specific expression indirectly by the synthesis of somatomedin (e.g. IGF I.), which when secreted from the liver would bind in an autocrine fashion to hepatic somatomedin receptors and stimulate the synthesis of male-specific proteins.

It is now well established that most membrane receptors that bind polypeptide hormones are down-regulated by chronic occupancy. The growth hormone receptor is unusual in that it is up-regulated by continuous growth hormone stimulation. Thus, any protein under direct control would be further stimulated by chronic exposure to growth hormone. This would account for the high levels of female specific proteins according to the Norstedt and Palmiter model.

Occupancy of the growth hormone receptor liberates a second messenger, as yet unidentified, which also acts to induce IGF I. This in turn binds to its hepatic receptor stimulating the production of proteins regulated by this pathway. In the female, the number of IGF receptors expected would be low due to continuous synthesis followed by chronic exposure of receptors. By contrast, in the male, the secretory bursts of growth hormone would presumably lead to a pulsatile emission of IGF I, hence IGF I receptor concentration would be higher in comparison to the female. An increase in receptor concentration would lead to an induction of those proteins controlled via this mechanism. a schematic diagram of this proposal is shown in figure 4.20

This hypothesis would fit well with findings obtained for the expression of the Yf subunit. In the little mouse, no growth hormone is present, this results in low levels of IGF I which in turn leads to a reduced expression of Yf. When growth hormone is administered to male and female little mice, IGF I is produced, which acting in an autocrine fashion stimulates the expression of the Yf subunit. In normal females, continuous secretion of growth hormone down-regulates the IGF receptor which results in a low level of Yf expression.

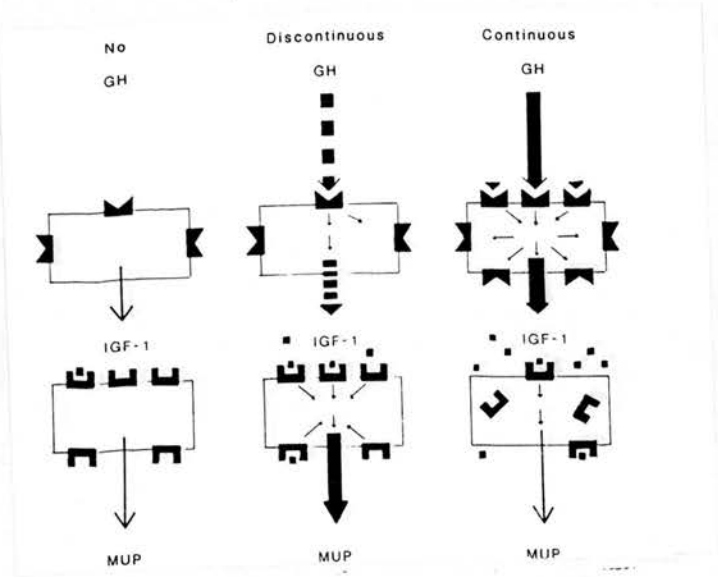


Figure 4.20 Hypothetical scheme for growth hormone action on sexually dimorphic hepatic proteins.

Liver cells exposed to different types of growth hormone stimulation. The figure to the left shows a situation where no growth hormone is present (e.g. little mice) and the resulting low production of IGF-I and the mouse major urinary protein (MUP) which is normally highly expressed in the male. The middle figure indicates a situation of discontinuous stimulation of growth hormone receptors (exemplified by male mice), which is postulated to result in discontinuous release of IGF-I, which in turn induces MUP. The figure to the right shows a continuous stimulation of growth hormone receptors (exemplified by female mice) which up-regulates growth hormone receptors, resulting in continuous IGF-I release, which in turn down regulates IGF-I receptors and results in low MUP production.

Very little evidence has been obtained to either confirm or deny this hypothesis. A recently developed culture system for hepatocytes in which the sexually differentiated expression of proteins is maintained during primary cell-culture will be a useful model to study this question (Guzelian *et al.*, 1988).

Using this system, induction of P-450 15β (female-specific) was observed in cultured male rat hepatocytes exposed to growth hormone. Isolated female hepatocytes required the continuous presence of growth hormone to maintain expression of 15β. Moreover, other hormones tested, androgens, oestrogens, IGF I, IGF II, did not induce P-450 15β levels in male or female hepatocytes. This demonstrates a direct effect of growth hormone on a female specific protein. The male-specific P-450 16α was not induced in either male or female hepatocytes with growth hormone. Other factors might be required for the induction of male-specific proteins or alternatively the pulsatile growth hormone pattern might not have been simulated properly.

Maitler *et al.*, (1988) have reported recently that IGF I and growth hormone receptor levels are under the control of the growth hormone secretory pattern. Intermittent administration of growth hormone leads to an increase in IGF I levels in hypophysectomised female rats (as compared to chronic exposure) no change in growth hormone receptor levels was observed. Continuous secretion of growth hormone leads to an increase in growth hormone receptor levels.

These observations, by no means confirming the scheme put forth by Norstedt and Palmiter (1984), suggest there might be some basis for this theory. Identification of responsive elements in growth hormone-regulated genes will hopefully allow for a clearer understanding of the mechanism of regulation and facilitate the isolation of

transacting factors involved in initiating transcription from both male- and female-specific genes. This together with the characterisation of the growth hormone receptor will provide powerful tools for uncovering the intracellular pathway of events which lead to sex-specific expression of hepatic proteins.

4.05d Further work

The treatment regime for growth hormone therapy would obviously have to be modified to achieve maximal expression of the Yf GST subunit in the little mouse. This type of experiment could also be applied to hypophysectomised mouse model to confirm the findings from this study. Growth hormone would be administered in both male and female patterns. It would also be predicted that other mouse models investigated during the course of this work would also be responsive to growth hormone therapy.

The pygmy mouse (King, 1950) might provide a very useful model to investigate the direct versus indirect action of growth hormone. The mutation is inherited as an autosomal recessive mapped to chromosome 10. Affected mice are approximately 50% the size of normal litter mates. Plasma prolactin and growth hormone levels have been found to be within the normal range (Sinha *et al.*, 1979). Nissley *et al.* (1980) reported that somatomedin production in the pygmy is normal. It is speculated that the underlying genetic defect in this mutant might be at the IGF receptor or in the transmission of the signal. Certainly, an investigation of the hepatic GST complement from these mice might provide a useful insight into the mechanism of growth hormone regulation of the Yf subunit.

Although the main focus of this work has been concerned with the endocrine regulation of the Yf subunit, it would appear from preliminary data that the Ya_1 subunit is also under the control of growth hormone. Hypophysectomy of both male and female mice leads to an increase in the levels of Ya_1. Little mice, again of both sexes, also display higher levels of this subunit than controls. This would suggest that the hepatic Ya_1 subunit is repressed by growth hormone, although a fuller investigation would have to be undertaken to confirm this finding. Other drug metabolising enzymes have been found to be suppressed by growth hormone (Waxman *et al.*, 1988).

A slight elevation in Yf has been noted in female mice after hypophysectomy. The Yf level in the male falls to that of the hypophysectomised female but not to that of the normal female. This would suggest that a hormone or hormones produced another endocrine gland is responsible for the suppression of expression in the liver, although due to the strong positive action of growth hormone this is not noted in the male. Strong possible candidates would be the adrenals or the thyroid gland. It would certainly be an interesting study in itself to explore the possible regulation of hepatic GST by other endocrine systems.

Chapter 5 - Pituitary Involvement In The Xenobiotic Control Of Mouse Hepatic GST

5.01 Introduction

The induction of drug-metabolising enzymes by foreign compounds has been extensively studied since the phenomenon was first described by Brown *et al.*, (1954). Particular emphasis has been placed on the cytochrome P-450 monooxygenase system, members of which can be induced by a vast array of foreign compounds. Indeed, until recently, when a new P-450 nomenclature was introduced (Nebert *et al.*, 1987), P-450 isoenzymes were classed on the basis of their regulation by foreign compounds. Four major xenobiotic inducible P-450 gene families have been described. The P-450 I gene family are inducible by polycyclic aromatic hydrocarbons (PAH), for example, 3-methycholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The most extensive family, the P-450 II gene family are inducible by phenobarbital (PB). The prototype inducer for the third gene family is pregnenolone 16α-carbonitrile (PCN), hence this class of P-450 are also referred to as the PCN-inducible P-450. This family, in the rat, can also be induced by steroids such as the synthetic glucocorticoid dexamethasone (Dex). Peroxisome proliferators, such as chlofibric acid, induce the forth gene family (for reviews, see Wolf, 1986; Nebert and Gonzalez, 1987)

Phenobarbital and polycyclic aromatic hydrocarbons (PAH) such as 3-MC have also been found to induce hepatic GST subunits for the alpha and mu classes in the rat (Kaplowitz *et al.*, 1975; Hayes *et al.*, 1979; Pickett *et al.*, 1987). Treatment of rats with these xenobiotics also leads to transcriptional activation of the corresponding genes (Ding and Pickett, 1985). Phenobarbital has also been reported to induce

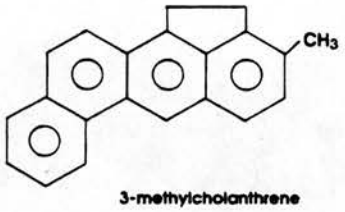
hepatic GST levels in the mouse, however this phenomenon has not been as well characterised at the molecular level in this species (Stockstill and Dauterman, 1982; David and Nerland, 1983; Davies and Schnell, 1987; Di Simplico *et al.*, 1989).

The aim of this study was to investigate the effect of phenobarbital (PB) and PB-like inducers on the hepatic GST profile in the mouse. The compounds phenobarbital, dexamethasone and 1,4-Bis [2-(3,5-dichloropyridyloxy)]-benzene (TCBOP) were investigated, the structures for which are given in fig 5.01. With respect to the induction of P-450 genes in the mouse, dexamethasone has been described as a potent phenobarbital-like inducer (Meechan *et al.*, 1988). TCBOP is again similar to PB in the induction of members of the mouse P-450 family. It has been estimated that TCBOP in the mouse is 650 times more potent at inducing P-450 than PB (Poland *et al.*, 1980). When tested on the rat however, this compound failed to illicit a response (Poland *et al.*, 1981). Thus, there can be profound differences between species in their response to foreign compounds.

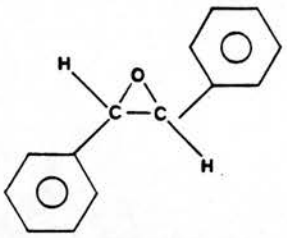
In the rat, the induction of members of the PB family by PB itself was enhanced after hypophysectomy. Removal of growth hormone was found to lead to a higher base-line level of expression of these genes which were further elevated after treatment (Yamazoe *et al.*, 1987). Thus, the possible effect of the pituitary gland, more specifically growth hormone, in the modulation of the xenobiotic regulation of mouse hepatic GST was also investigated. An analysis of the effect of the xenobiotics listed above in hypophysectomised and "little" mouse models was undertaken.

Figure 5.01

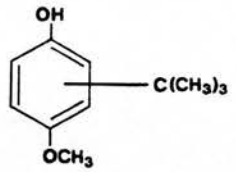
Structures of GST inducing xenobiotic compounds.



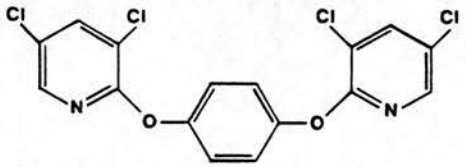
3-methylcholanthrene



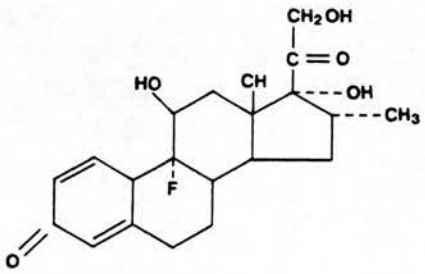
trans - stilbene oxide



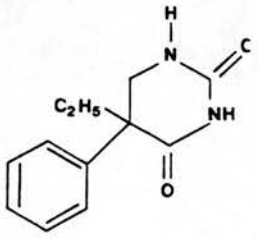
butylated hydroxyanisole



TCBOP



DEXAMETHASONE



PHENOBARBITAL

5.02 Effect of xenobiotic treatment on hepatic GST content in C57BL/6 and DBA/2 mice

5.02a Animal treatment

Male and female mice of the C57BL/6 and DBA/2 strains (4 per group) were treated with the xenobiotic compounds phenobarbital, dexamethasone and TCBOP. Phenobarbital (in saline) and dexamethasone (in corn oil) were administered interperitoneally at 80mg/kg/day for 3 days and 100mg/kg/day for 4 days respectively. Mice were sacrificed 24 hours after the cessation of treatment. One interperitoneal injection of TCBOP (3mg/kg) was administered and the animals killed 4 days after treatment. Control animals received corn oil. After sacrifice livers were removed and rapidly frozen on dry-ice. Tissues were stored at -70°C.

The effect of a single injection of TCBOP (3mg/kg) was monitored over a 12 week period in both the C57BL/6 and DBA/2 strains, both sexes were investigated. Two mice per group were sacrificed at 2, 4, 8 and 12 weeks after the initial injection.

5.02b Analysis of hepatic GST content after xenobiotic treatment in the C57BL/6 and DBA/2 strains of mice.

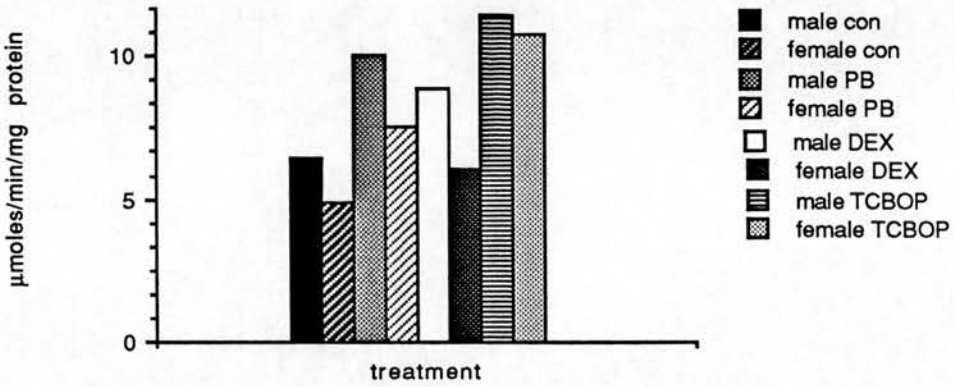
Cytosols were prepared from pooled liver samples for each of the treatment groups described above. Specific activities were determined for CDNB, the general GST substrate and DCNB which is the substrate relatively specific for the Yb_1 subunit. Activity towards these substrates was higher after xenobiotic treatment in both strains and sexes. Of the xenobiotics studied, TCBOP was the most potent inducer causing a 5-fold increase in DCNB activity (figures 5.02, 5.03). No difference in

Figure 5.02

Levels of CDNB activity in hepatic cytosols from male and female C57BL/6 and DBA/2 mice treated with xenobiotic.

Figure 5.02

a. CDNB activity - male and female C57BL/6 mice



b. CDNB activity - male and female DBA/2 mice

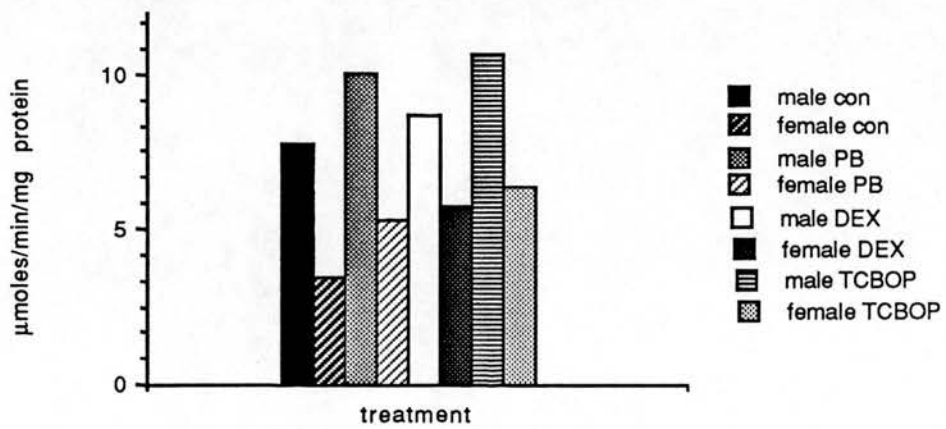
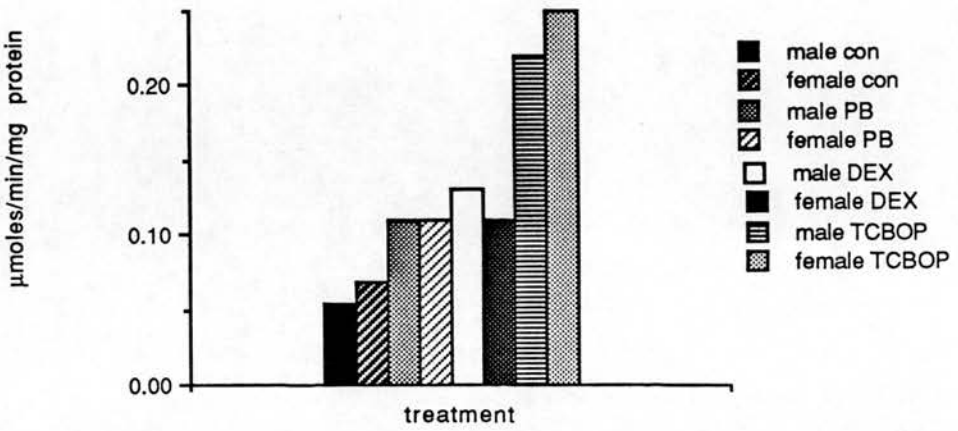


Figure 5.03

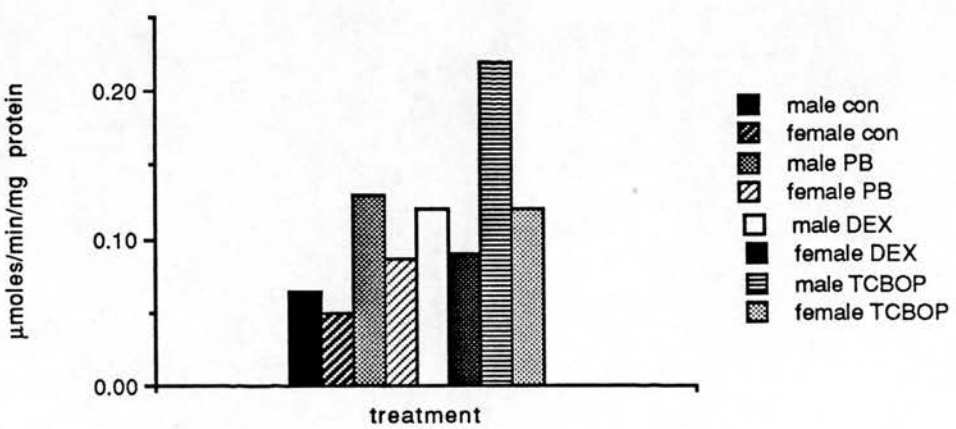
Levels of DCNB activity in hepatic cytosols from male and female C57BL/6 and DBA/2 strains of mice after treatment with xenobiotic.

Figure 5.03

a. DCNB activity - C57BL/6 mice



b. DCNB activity - DBA/2 mice



DCNB was detected between males and females of the C57BL/6 strain. Levels of DCNB activity were consistently lower in females of the DBA/2 strain as compared to the males in both control and xenobiotic treated groups. The sex difference in the expression of the Yf subunit was reflected in the CDNB activity, females were consistently lower than males in both control and treated groups.

SDS/PAGE followed by western blotting with antisera raised against purified mouse GST demonstrated that members of the Yb gene family were induced by xenobiotic treatment, this occured in both strains and sexes (figures 5.04, 5.05). Phenobarbital and dexamethasone induced mu class GST to a similar extent (approximately 2-3-fold). Again, TCBOP was the most potent inducer raising the levels of the Yb class approximately 5-fold. Possible changes in the expression of Yf and the Ya class subunits were not investigated since SDS/PAGE revealed that polypeptides of Mr 25,800 (Ya_3) and 24,800 (Yf) did not change markedly after the administration of these compounds. In the future, a more rigourous analysis of hepatic GST content will have to be undertaken to characterise fully the changes which occur in the expression of these enzymes following administration of foreign compounds. From the compounds investigated, preliminary results would indicate that it is the Yb gene family in the mouse which is most affected by treatment with xenobiotics.

The longer term effects of TCBOP treatment on hepatic GST content were investigated. After treatment of both strains and sexes with a single interperitoneal injection of TCBOP at a dose of 3mg/kg, CDNB and DCNB activity reached maximum levels 2-4 weeks after treatment, falling gradually back to basal levels. However, even after 12 weeks, activity had not completely declined to that observed in the controls. Induction was greatest in the C57BL/6 strain (figures 5.06, 5.07). Changes observed in the

Figure 5.04

Electrophoretic analysis of male and female C57BL/6 and DBA/2 mice treated with xenobiotics.

Cytosols (30μg) from pooled liver samples were analysed by SDS/PAGE on a 12% (w/v) polyacrylamide resolving gel. Gel loadings were as follows :- lanes 1and 10, rat lung standards (Yc, Yb, Yf); lane 2, control male; lane 3, control female; lane 4, male treated with PB; lane 5, female treated with PB; lane 6, male treated with DEX; lane 7, female treated with DEX; lane 8, male treated with TCBOP; lane 9, female treated with TCBOP lane 10, rat liver standards (Yc, Yb, Ya).

Panel a. C57BL/6.

Panel b. DBA/2.

Figure 5.04a

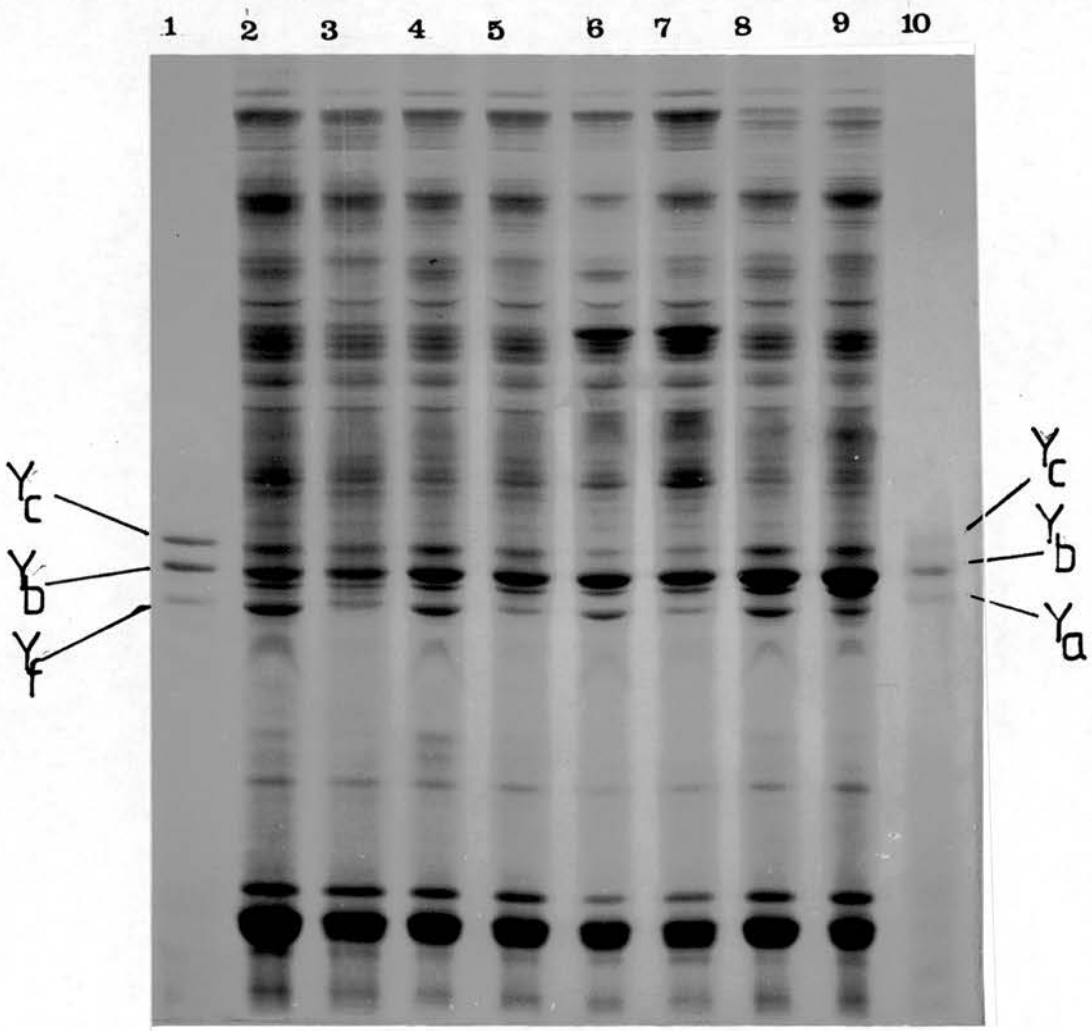


Figure 5.04

Electrophoretic analysis of male and female C57BL/6 and DBA/2 mice treated with xenobiotics.

Cytosols (30μg) from pooled liver samples were analysed by SDS/PAGE on a 12% (w/v) polyacrylamide resolving gel. Gel loadings were as follows :- lanes 1and 10, rat lung standards (Yc, Yb, Yf); lane 2, control male; lane 3, control female; lane 4, male treated with PB; lane 5, female treated with PB; lane 6, male treated with DEX; lane 7, female treated with DEX; lane 8, male treated with TCBOP; lane 9, female treated with TCBOP; lane 10, rat liver standards (Yc, Yb, Ya).

Panel a. C57BL/6.

Panel b. DBA/2.

Figure 5.04b

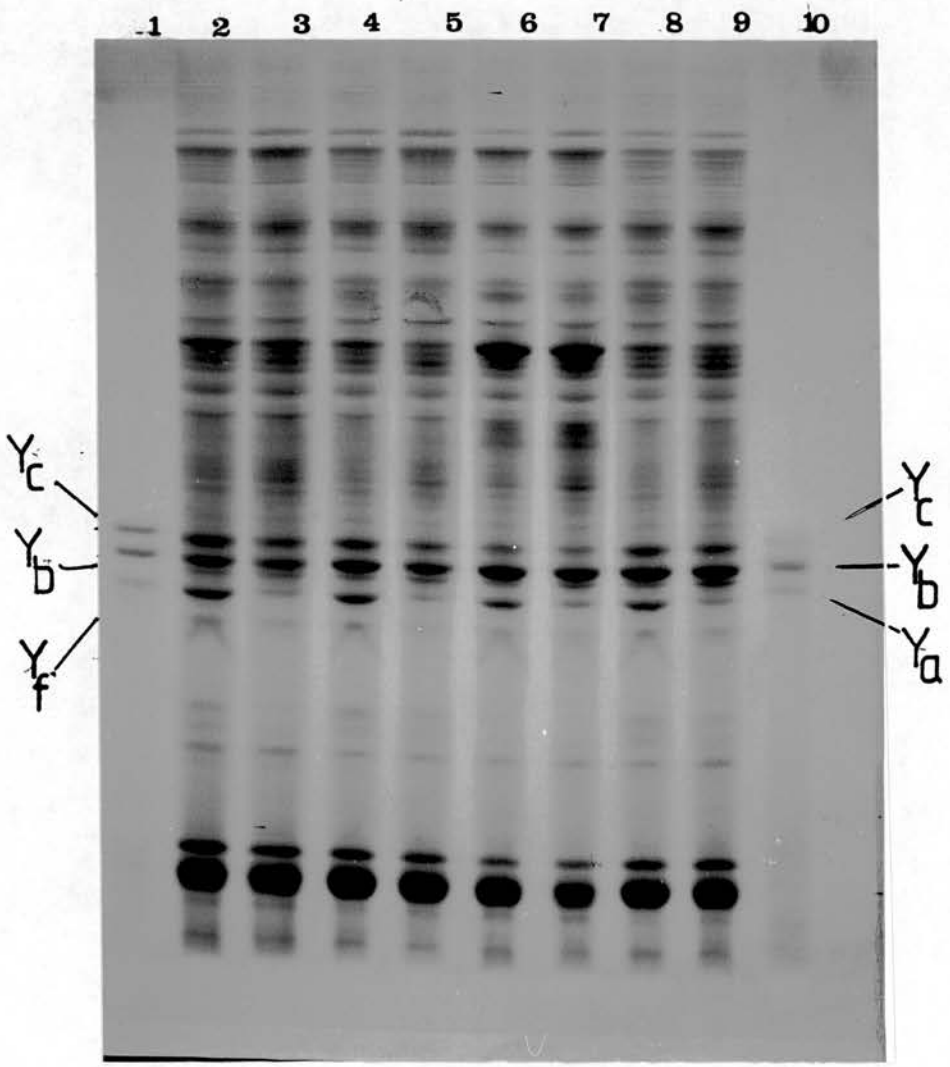


Figure 5.05

Immunoblotting of hepatic cytosols from male and female C57Bl/6 and DBA/2 strains using anti-(mouse Yb_1Yb_1)antisera.

Cytosols (50μg) were subject to SDS/PAGE on a 12% (w/v) polyacrylamide resolving gel, followed by transfer to nitrocellulose and immunoblotting with anti-(rat Yb_1Yb_1) IgG. The gel was loaded as follows :- lanes 1 and 10, rat lung standards (Yc, Yb, Ya); lane 2, male control; lane 3, female control; lane 4, male treated with PB; lane 5, female treated with PB; lane 6, male treated with DEX; lane 7, female treated with DEX; lane 8, male treated with TCBOP; lane 9, female treated with TCBOP.

Panel a. C57BL/6 mice

Panel b. DBA/2 mice.

Figure 5.05

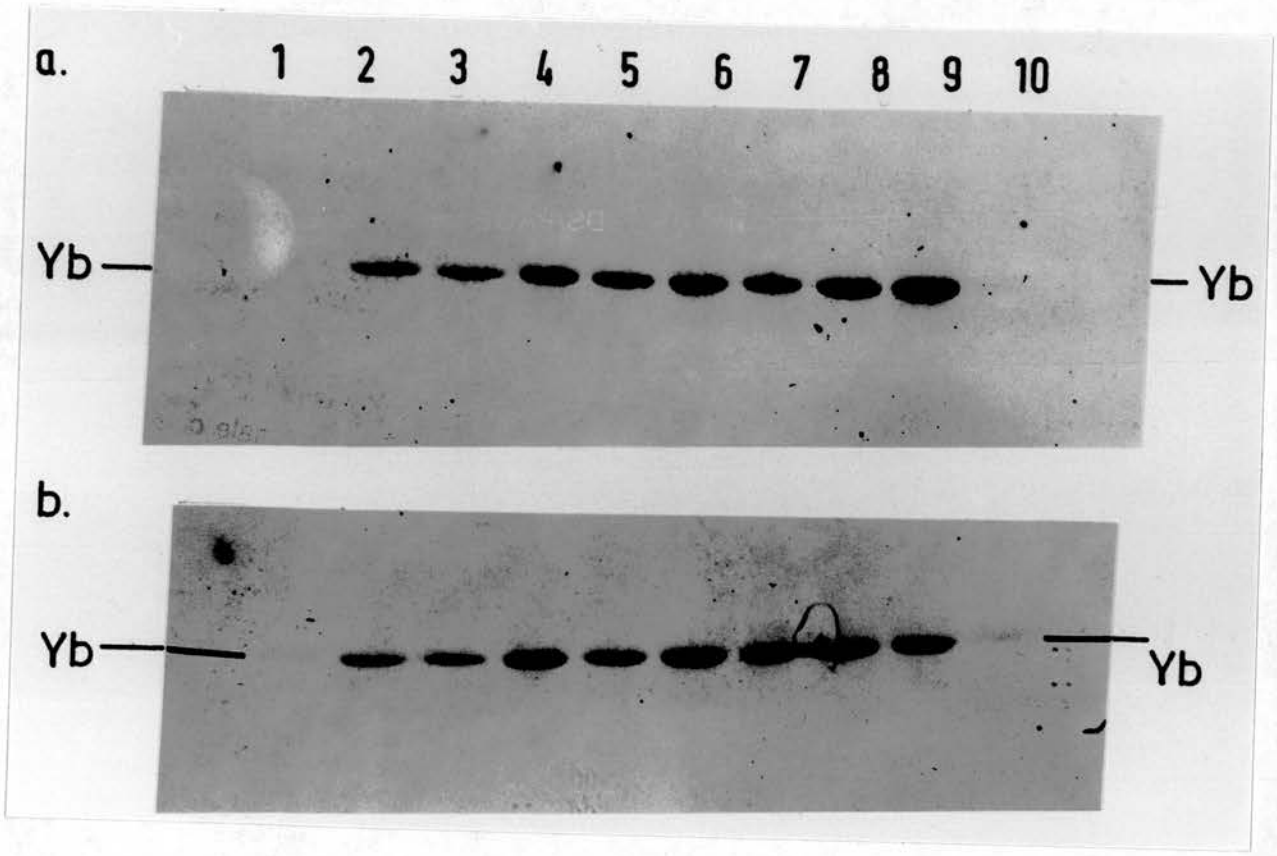
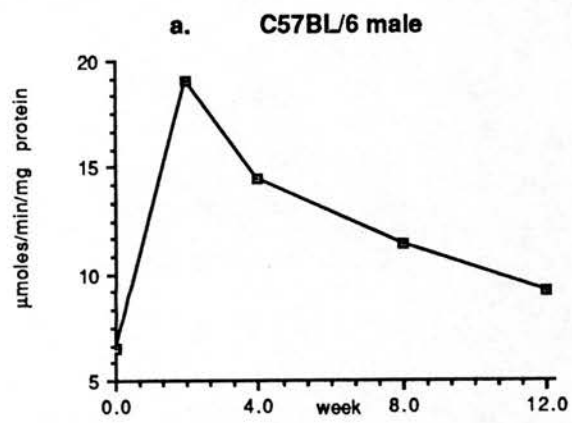
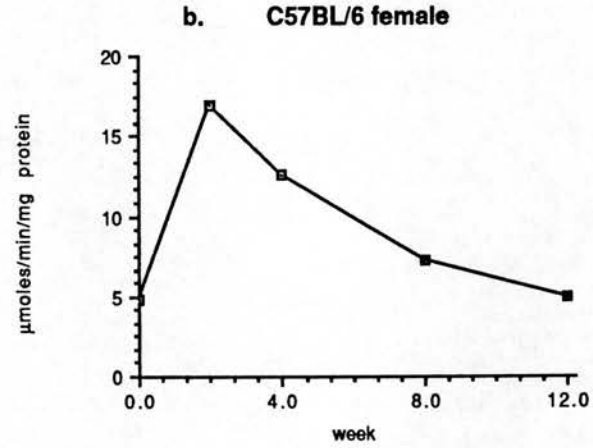


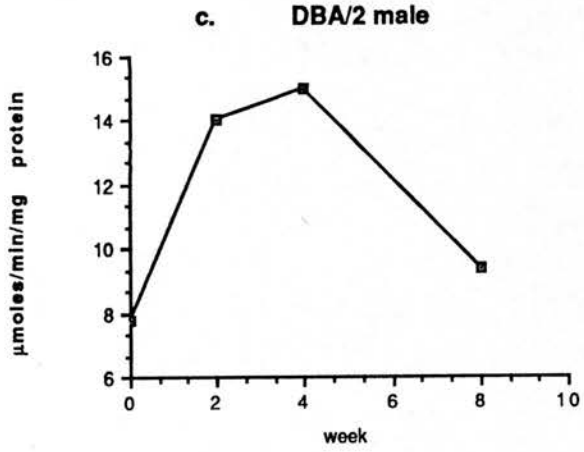
Figure 5.06

Levels of CDNB activity in C57BL/6 and DBA/2 mice over a 12 week period after treatment with TCBOP

Figure 5.06


a.
C57BL/6 male
µmoles/min/mg protein
20
15
10
5
0.0
4.0
week
8.0
12.0


b.
C57BL/6 female
µmoles/min/mg protein
20
15
10
5
0
0.0
4.0
8.0
12.0
week


c.
DBA/2 male
µmoles/min/mg protein
16
14
12
10
8
6
0
2
4
6
8
10
week

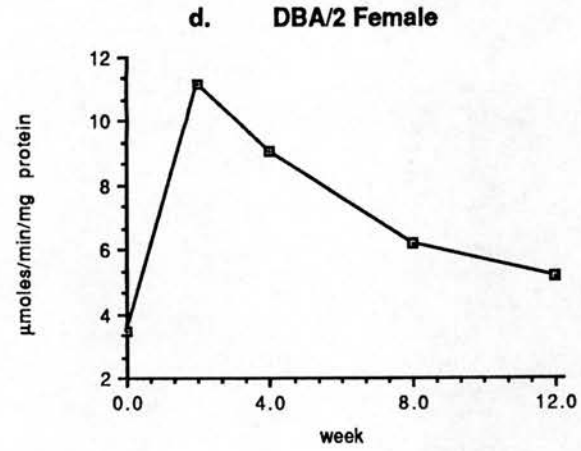
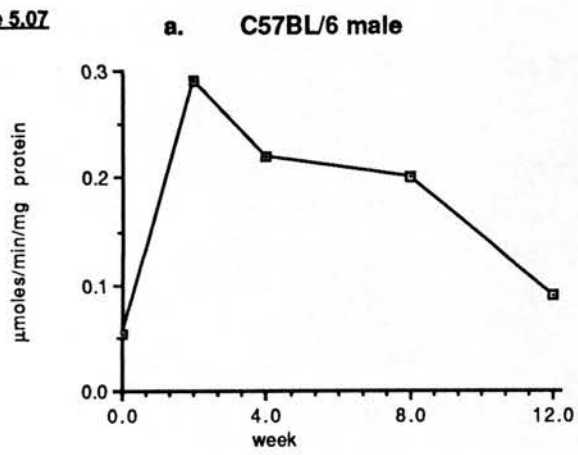
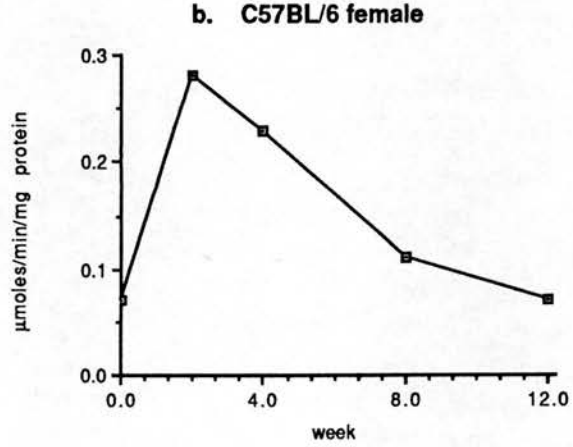

d.
DBA/2 Female
µmoles/min/mg protein
12
10
8
6
4
2
0.0
4.0
8.0
12.0
week

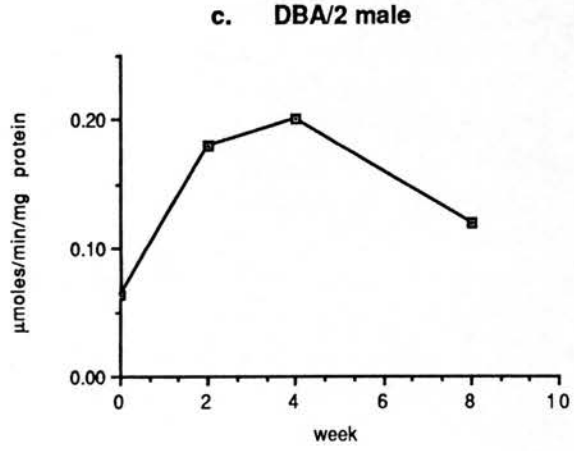
Figure 5.07

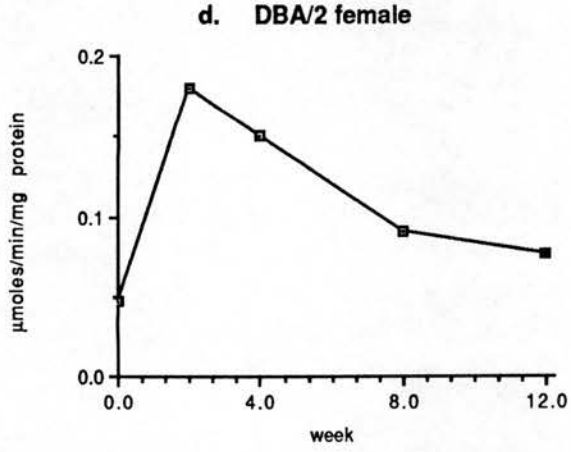
Level of DCNB activity in C57BL/6 and DBA/2 mice over a 12 week period after treatment with TCBOP

Figure 5.07


a. C57BL/6 male
µmoles/min/mg protein
0.3
0.2
0.1
0.0
0.0
4.0
8.0
12.0
week


b. C57BL/6 female
µmoles/min/mg protein
0.3
0.2
0.1
0.0
0.0
4.0
8.0
12.0
week


c. DBA/2 male
µmoles/min/mg protein
0.20
0.10
0.00
0
2
4
6
8
10
week


d. DBA/2 female
µmoles/min/mg protein
0.2
0.1
0.0
0.0
4.0
8.0
12.0
week

levels of Yb-type subunits reflected the changes described for CDNB and DCNB (figures 5.08, 5.09). Maximum induction, with the exception of the DBA/2 males, occured at the 2-week time-point, the mu class GST being elevated approximately 7 to 8-fold.

5.03 Effect of hypophysectomy on xenobiotic induction of hepatic GST in C57BL/6 mice.

5.03a Animal treatment

Males and females of the C57BL/6 strain, hypophysectomised or sham operated at 8 weeks of age, were obtained from Charles Rivers Laboratories, Willmington, Massachusetts. After transportation the animals were allowed to settle for 2 weeks prior to the commencement of treatment. After acclimatization, animals were treated with PB, DEX or TCBOP (2-4 animals per group). Phenobarbital (in saline) was injected interperitoneally at a dose of 80mg/kg/day for 3 days. Dexamethasone (in corn oil) was administered at 100mg/kg/day for 4 days. Animals were sacrificed 24 hours after the final treatment session. One interperitoneal injection of TCBOP (in corn oil) was administered and the animals were killed on the fourth day after treatment. Control animals received corn oil. Information on the treatment groups and the number of animals per group is given in table 5.01. Tissues were removed directly after sacrifice, frozen on dry-ice and stored at -70°C.

Plasma thyroxine (T4) levels in control and hypophysectomised were measured according to the method of Ratcliffe *et al.* (1974) to ensure that the operative procedure had been successful (figure 5.10). However, treatment of mice with xenobiotic compounds would appear to affect plasma T4 levels Treatment with TCBOP, for example, causes a sharp decline in T4 levels in sham operated animals. The mechanism underlying this finding is not known. It may be that xenobiotic compounds affect the production and/or secretion of thyroid hormones from the thyroid gland. Alternatively, xenobiotics compounds may affect the subsequent metabolism of the thyroid hormones. Measurement of body weight changes would in this case constitute a more reliable method of assessing the success of hypophysectomy.

Figure 5.08

Electrophoretic analysis of hepatic cytosols from a). C56Bl/6 mice and b). DBA/2 mice treated with TCBOP : a time course.

Cytosols (30μg) from pooled liver samples were analysed by SDS/PAGE on a 12% (w/v)polyacrylamide gel. The gel was loaded as follows :- lane 1, rat lung standards; lane 2, control male; lane 3, control female; lane 4, male treated with TCBOP, 2 week time point; lane 5, female treated with TCBOP : 2 week time point; lane 6, male treated with TCBOP, 4 week time point; lane 7, female treated with TCBOP : 4 week time point; lane 8, male treated with TCBOP : 8 week time point; lane 9, female treated with TCBOP : 8 week time point; lane 10, male treated with TCBOP : 12 week time point; lane 11, female treated with TCBOP : 12 week time point. lane 12, purified mouse Yf GST.

Panel a. C57BL/6.

Panel b. DBA/2.

Figure 5.08

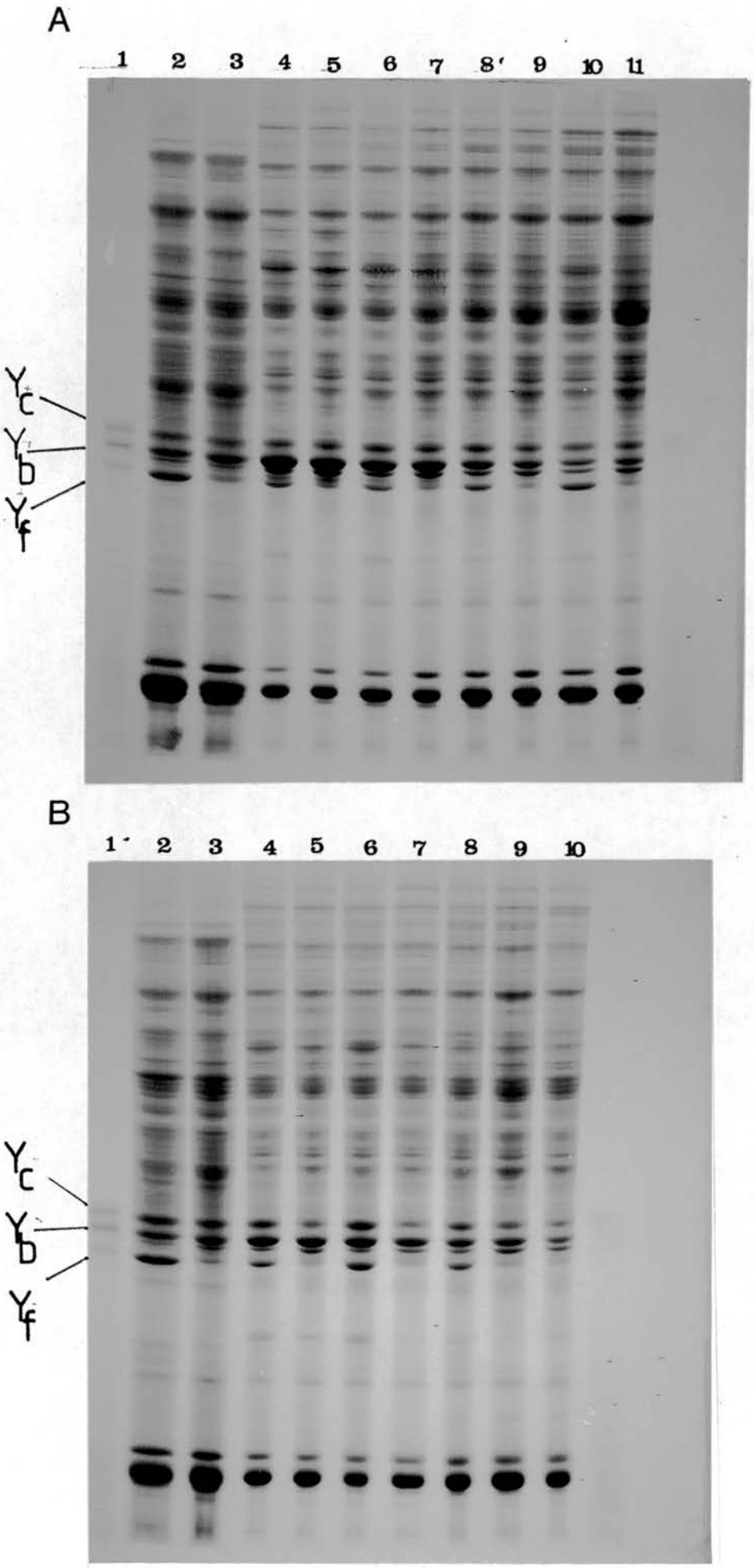


Figure 5.09

Immunoblotting of hepatic cytosols from a). C57BL/6 mice and b). DBA/2 mice treated with TCBOP : a time course.

Pooled cytosols (50μg) were subject to SDS/PAGE, transferred to nitrocellulose and blotted against anti-(rat Yb_1Yb_1)IgG. The gel was loaded as follows :- lanes 1 and 12, rat lung standards (Yc, Yb, Yf); lane 2, control male; lane 3, control female; lane 4, male 2 weeks TCBOP treatment; lane 5, female 2 weeks TCBOP treatment; lane 6, male 4 weeks TCBOP treatment; lane 7, female TCBOP treatment 4 weeks; lane 8, male TCBOP treatment 8 weeks; lane 9

A. C57BL/6 Strain.

B. DBA/2 Strain.

Figure 5.09

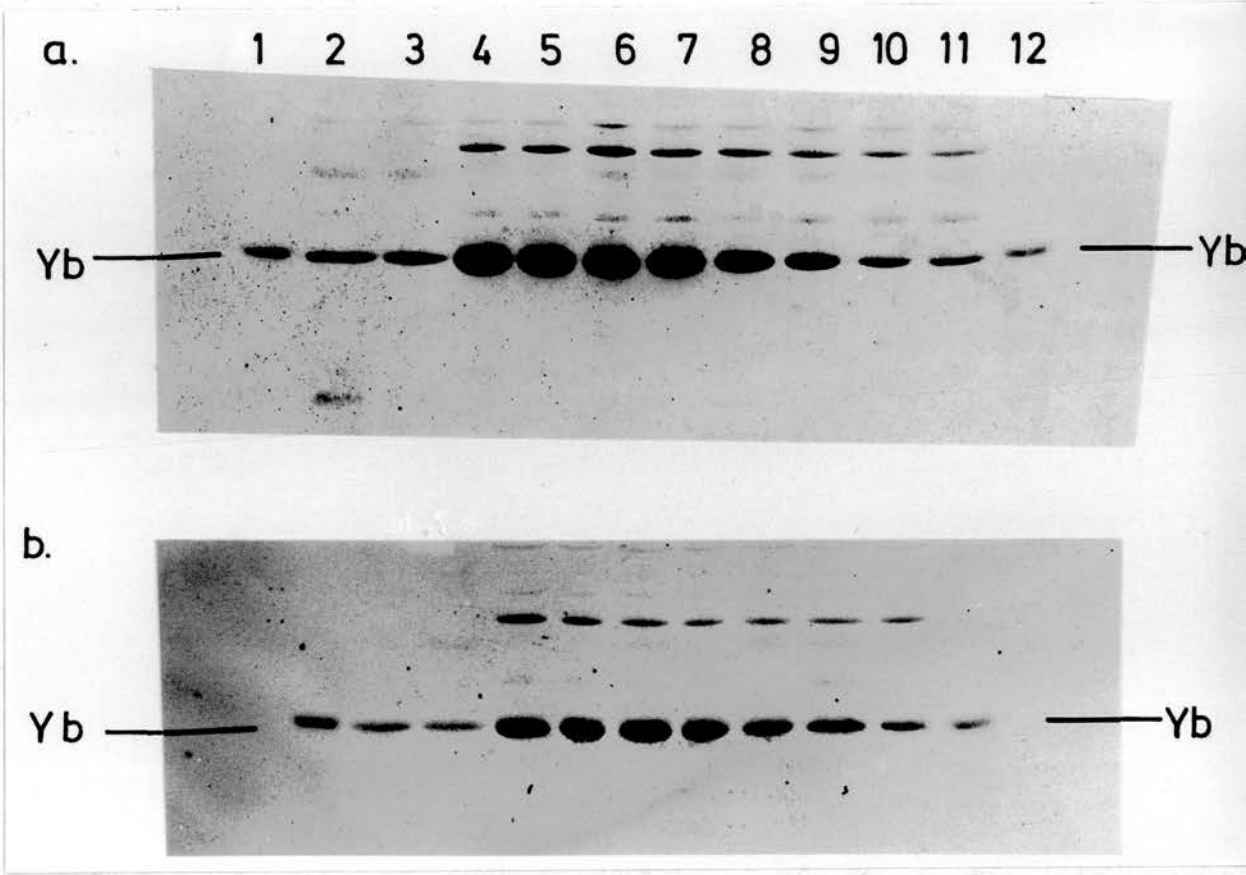
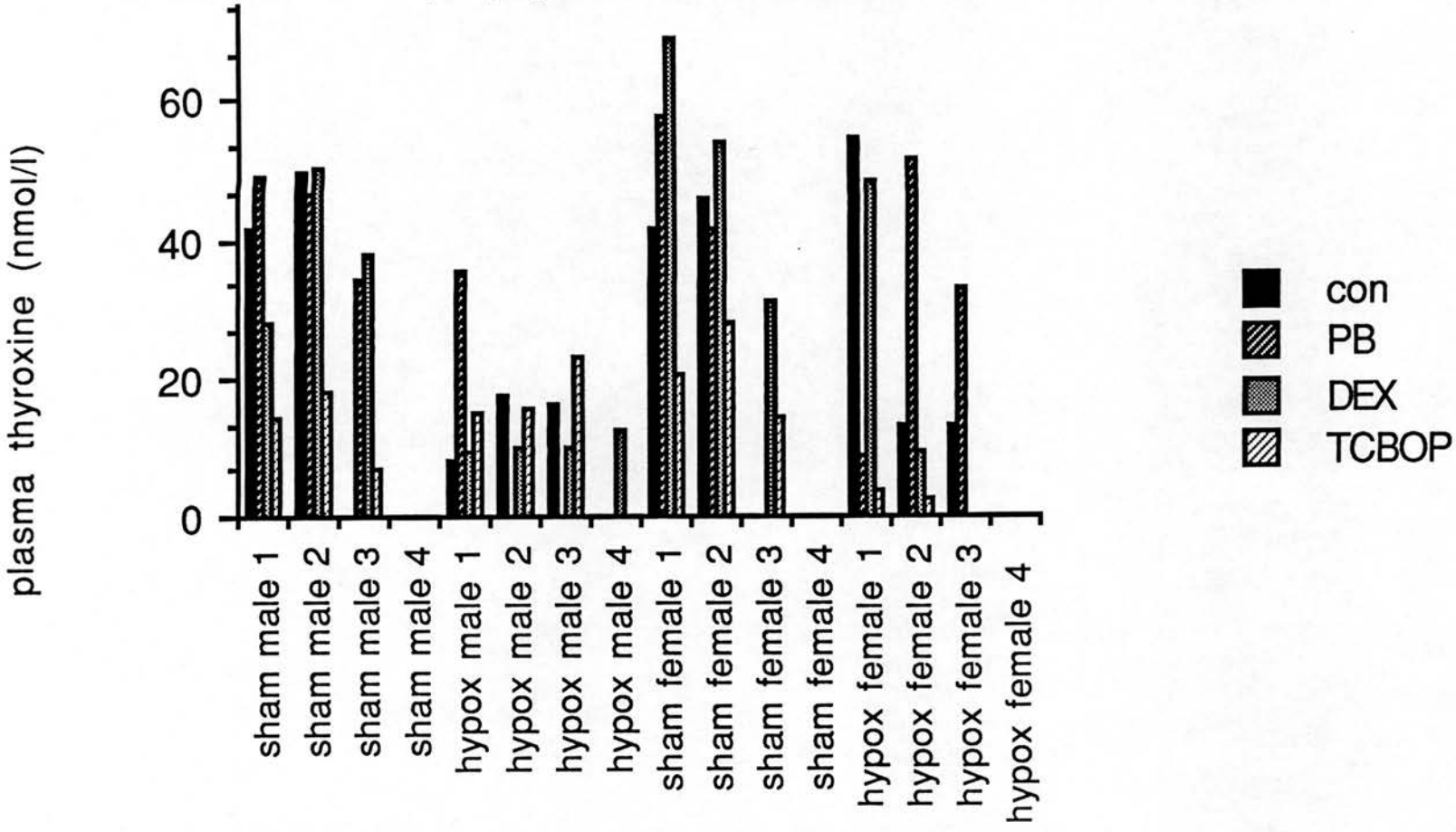


Table 5.01 - A summary of treatments groups for experimental section 5.03a

Operative status	Sex	Treatment	Group number
Sham	male	corn oil	2
Sham	female	corn oil	2
Hypox	male	corn oil	3
Hypox	female	corn oil	3
Sham	male	PB	3
Sham	female	PB	2
Hypox	male	PB	4
Hypox	female	PB	4
Sham	male	DEX	3
Sham	female	DEX	3
Hypox	male	DEX	4
Hypox	female	DEX	4
Sham	male	TCBOP	3
Sham	female	TCBOP	3
Hypox	male	TCBOP	4
Hypox	female	TCBOP	4

Abbreviations : hypox - hypophysectomy; PB - phenobarbital; DEX - dexamethasone; TCBOP - 1,4-bis [2-(3,5-dichloropyridyloxy)]-benzene.

Figure 5.10
Plasma T4 levels in hypophysectomised and sham operated controls



For some mice no plasma was obtained, these are indicated by the spaces which occur in the figure.

5.03b Analysis of hepatic GST content.

Figure 5.11 presents, in histogram form, the CDNB activities for control and hypophysectomised animals treated with inducing agents detailed in section 5.03a. An increase in CDNB activity of approximately 1.5-fold is observed in sham operated groups of both sexes on treatment with these compounds. A decline in CDNB activity was observed after the removal of the pituitary in both control and treated groups. This most probably reflects the drop in the Yf subunit which occurs after hypophysectomy (refer to section 4.02).

SDS/PAGE and western blotting with anti-mouse GST antisera reconfirmed findings detailed in section 5.02b. Phenobarbital and dexamethasone cause an induction in Yb-type subunits, TCBOP again was found to be the most powerful inducer (causing at least a 5-fold induction of mu class gene family members). The constitutively expressed Ya subunit (Ya_3) was not significantly affected. Removal of the pituitary gland does not appear to influence significantly the induction of the Yb-type subunits by these xenobiotics. However, a slight elevation in the level of the Yb class is observed on hypophysectomy.Treatment with TCBOP was observed to increase the expression of Ya_1 in the livers of male and female mice. Levels of this subunit were also elevated after hypophysectomy as was also shown in section 4.02. Both treatments combined led to a further increase in the expression of this subunit (figures 5.12, 5.13). The effects of phenobarbital and dexamethasone treatment in relation to this subunit were not investigated. Hypophysectomy of male mice, as has been demonstrated previously, leads to a marked decline in the normally high expression of the Yf subunit. Interestingly, in hypophysectomised males treated with TCBOP Yf levels do not drop to that observed in other hypophysectomised groups both control and treated.

Figure 5.11

Levels of CDNB activity in hypophysectomised and sham operated animals treated with xenobiotics.

a.) phenobarbital

b). dexamethasone

c). TCBOP.

Figure 5.11

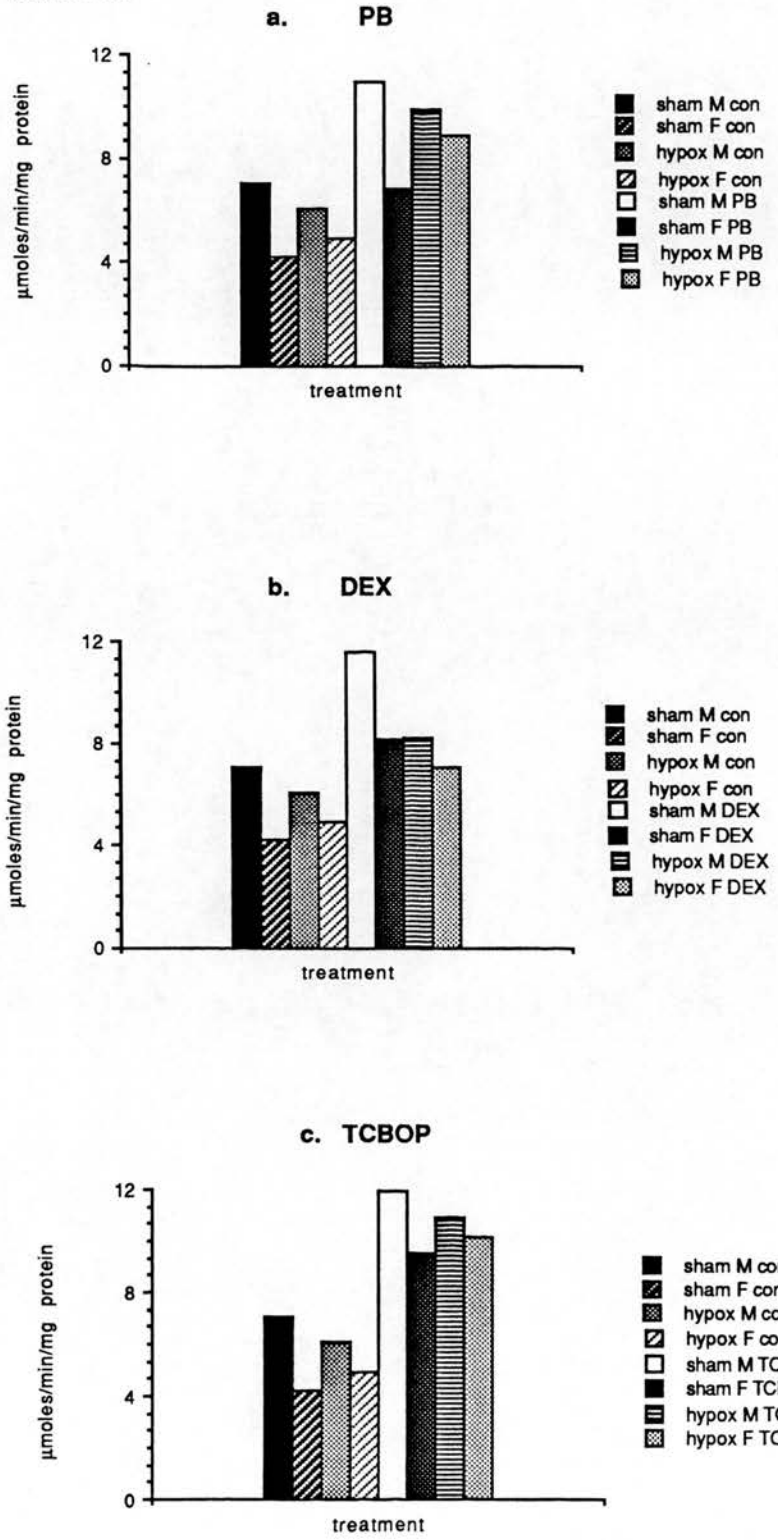

a. PB
12
8
4
0
µmoles/min/mg protein
treatment
sham M con
sham F con
hypox M con
hypox F con
sham M PB
sham F PB
hypox M PB
hypox F PB
b. DEX
12
8
4
0
µmoles/min/mg protein
treatment
sham M con
sham F con
hypox M con
hypox F con
sham M DEX
sham F DEX
hypox M DEX
hypox F DEX
c. TCBOP
12
8
4
0
µmoles/min/mg protein
treatment
sham M con
sham F con
hypox M con
hypox F con
sham M TCBOP
sham F TCBOP
hypox M TCBOP
hypox F TCBOP

Figure 5.12

Elecrophoretic analysis of hepatic cytosols from sham operated and hypophysectomised animals treated with xenobiotic.

Pooled cytosols (30μg) were analysed by SDS/PAGE on a 12% polyacrylamide resolving gel. Loadings were as follows :-

Figure 5.12a

lanes 1 and 15, rat liver markers; lane 2, mouse Yf standard; lane 3, sham male; lane 4, sham female; lane 5, hypox male; lane 6, hypox female; lane7, sham male treated with DEX; lane 8, sham female treated with DEX; lane 9, hypox male treated with DEX; lane 10, hypox female treated with DEX; lane 11, sham male treated with TCBOP; lane 12, sham female treated with TCBOP; lane 13, hypox male treated with TCBOP; lane 14, hypox female treated with TCBOP.

Figure 5.12b

lanes 1 and 12, rat liver standards; lanes 2 and 11, mouse Yf marker; lane 3, sham male; lane 4, sham female; lane 5, hypox male; lane 6 hypox female; lane 7 sham male treated with PB; lane 8, sham female treated with PB; lane 9, hypox male treated with PB; lane 10, hypox female treated with PB.

Figure 5.12

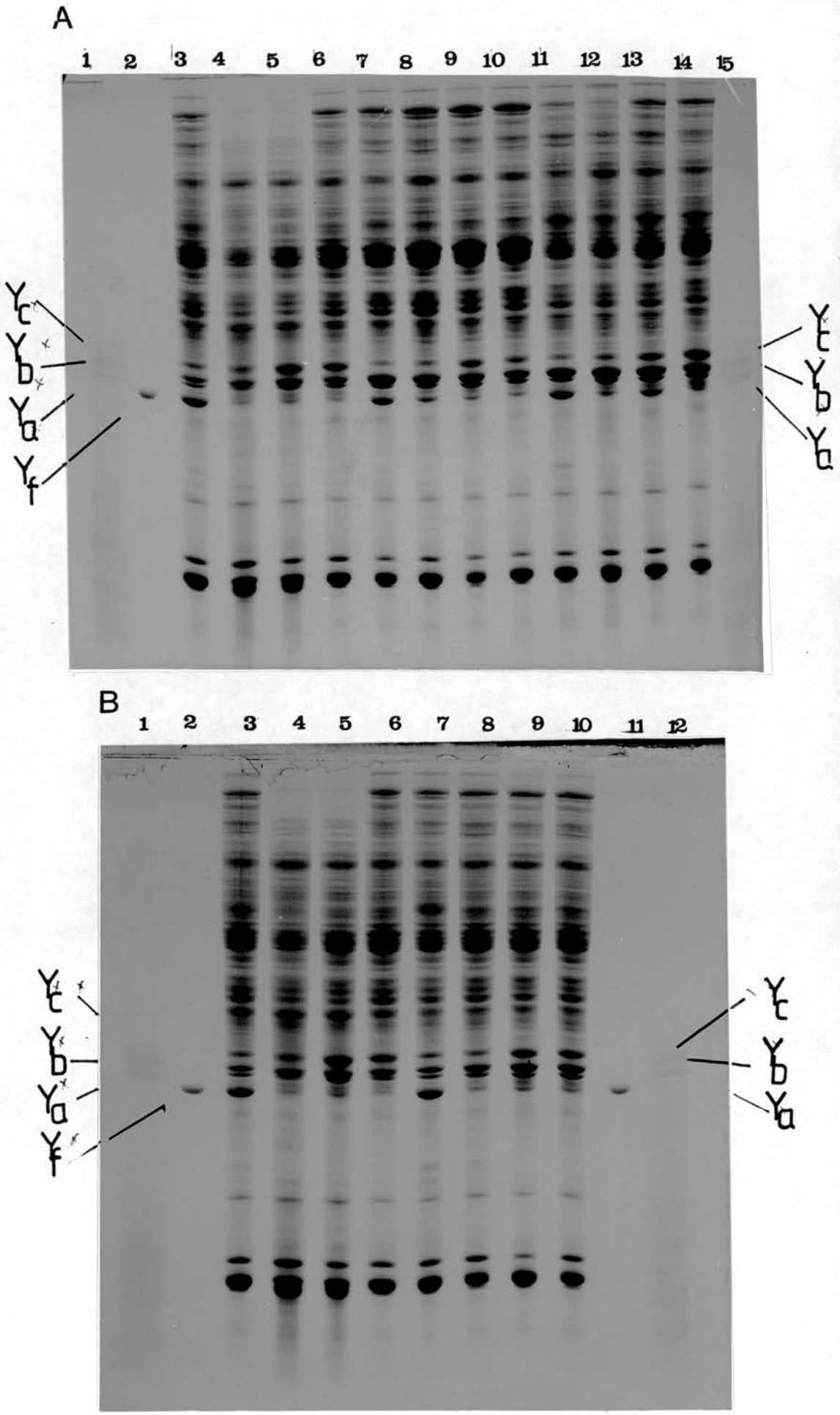

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Yc
Yb
Ya
Yf
Yc
Yb
Ya
B
1 2 3 4 5 6 7 8 9 10 11 12
Yc
Yb
Ya
Yf
Yc
Yb
Ya

Figure 5.13

Immunoblotting of hepatic cytosols from sham operated or hypophysectomised mice treated with xenobiotics.

Pooled cytosols (50μg) were subject to SDS/PAGE, transfered to nitrocellulose and immunoblotted with antimouse GST antisera. Gel loadings were as follows : - lanes 1 and 10, GST markers; lane 2, sham male; lane 3, sham female; lane 4, hypox male; lane 5, hypox female; lane 6, sham male treated with xenobiotic; lane 7 sham female treated with xenobiotic; lane 8, hypox male treated with xenobiotic; lane 9, hypox female treated with xenobiotic.

No. 1 - control and PB treated animals.

No. 2 - control and DEX treated animals.

No. 3 - control and TCBOP treated animals.

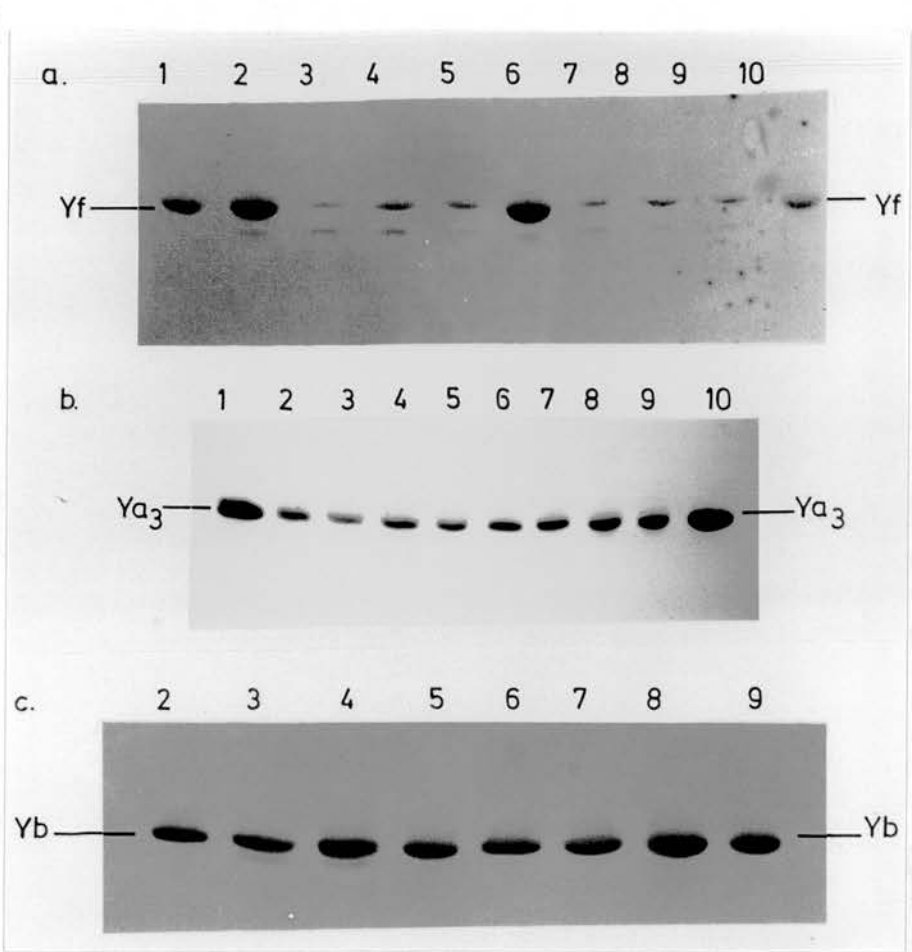
Panel a. anti-(mouse YfYaf) IgG.

Panel b. anti-(mouse Ya_1Ya_1) IgG.

Panel c. anti-(mouse Ya_3Ya_3) IgG.

Panel d. anti-(mouse Yb_1Yb_1) IgG.

Figure 5.13


1
a.
1 2 3 4 5 6 7 8 9 10
Yf
Yf
b.
1 2 3 4 5 6 7 8 9 10
Ya3
Ya3
c.
2 3 4 5 6 7 8 9
Yb
Yb

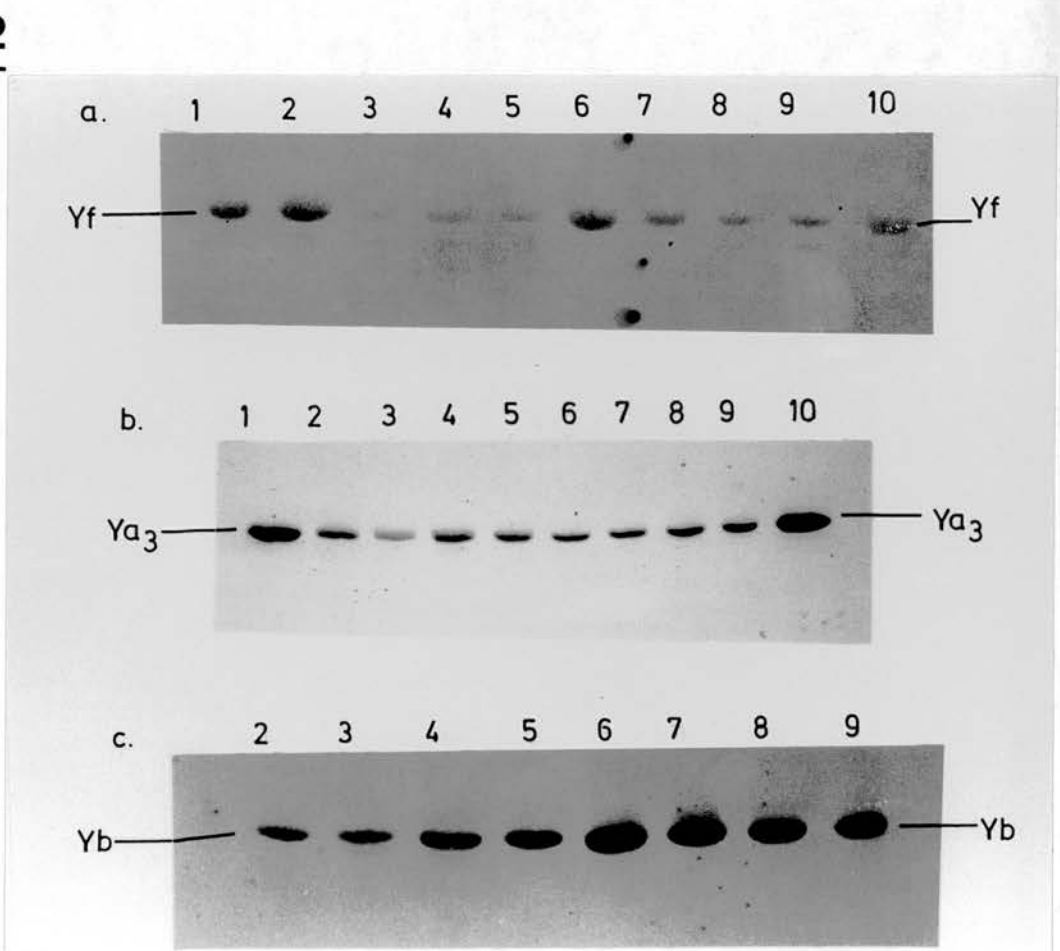

2
a.
1 2 3 4 5 6 7 8 9 10
Yf
Yf
b.
1 2 3 4 5 6 7 8 9 10
Ya3
Ya3
c.
2 3 4 5 6 7 8 9
Yb
Yb

Figure 5.13

Immunoblotting of hepatic cytosols from sham operated or hypophysectomised mice treated with xenobiotics.

Pooled cytosols (50μg) were subject to SDS/PAGE, transfered to nitrocellulose and immunoblotted with antimouse GST antisera. Gel loadings were as follows : - lanes 1 and 10, GST markers; lane 2, sham male; lane 3, sham female; lane 4, hypox male; lane 5, hypox female; lane 6, sham male treated with xenobiotic; lane 7 sham female treated with xenobiotic; lane 8, hypox male treated with xenobiotic; lane 9, hypox female treated with xenobiotic.

No. 1 - control and PB treated animals.

No. 2 - control and DEX treated animals.

No. 3 - control and TCBOP treated animals.

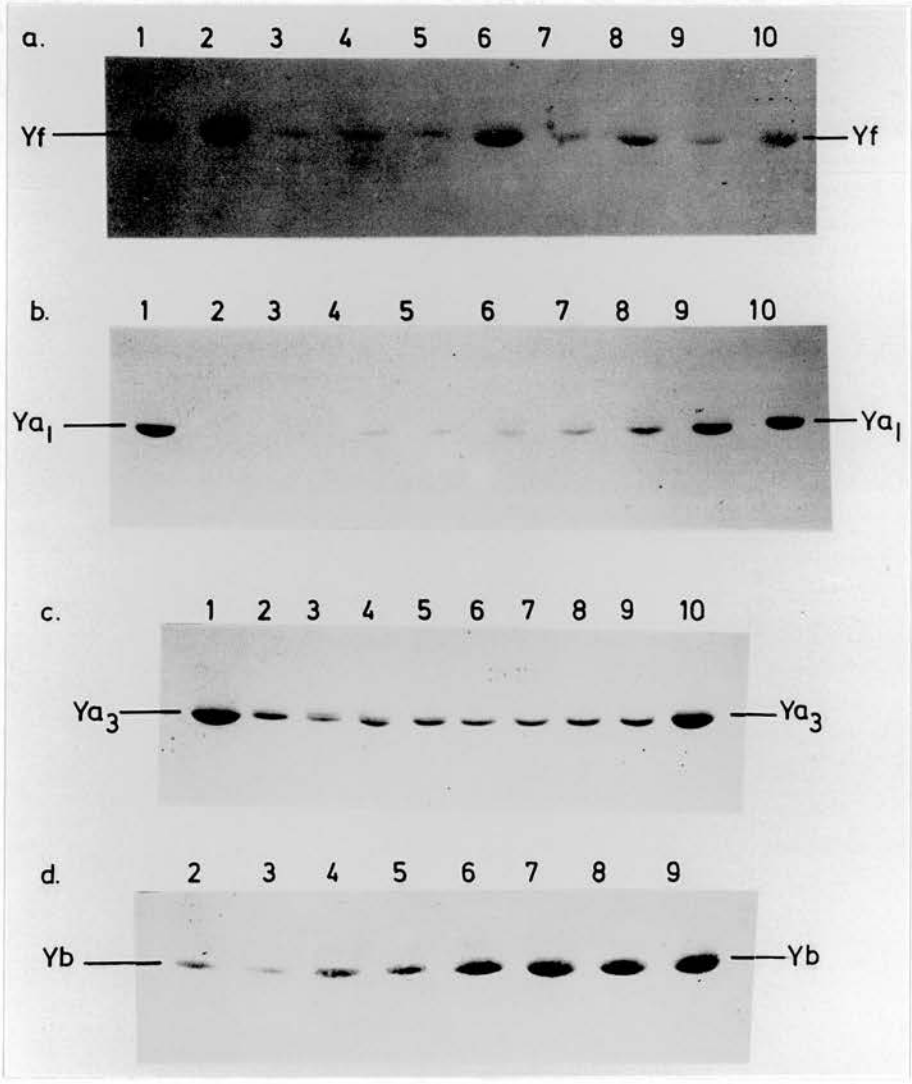
Panel a. anti-(mouse YfYaf) IgG.

Panel b. anti-(mouse Ya_1Ya_1) IgG.

Panel c. anti-(mouse Ya_3Ya_3) IgG.

Panel d. anti-(mouse Yb_1Yb_1) IgG.

Figure 5.13


3
a.
1 2 3 4 5 6 7 8 9 10
Yf
Yf
b.
1 2 3 4 5 6 7 8 9 10
Ya1
Ya1
c.
1 2 3 4 5 6 7 8 9 10
Ya3
Ya3
d.
2 3 4 5 6 7 8 9
Yb
Yb

Unfortunately TCBOP also causes a decline in plasma T4 levels in intact animals. It is therefore not possible to determine if the operative procedure was carried out correctly and the pituitaries completely removed from the animals within this group.

Northern blot analysis with the rat GST **cDNA probes** described in section 2.12b demonstrated that the changes observed at the protein level are also reflected at the mRNA level (figure 5.14).

McLellan and Hayes (1989) characterised a novel alpha class subunit (Ya_1) in the mouse, inducible by the dietary additive BHA, which by several criteria is more closely related to the rat Ya subunit than the constitutively expressed Ya_3 (see sections 1.07,1.08. Pearson *et al.* (1988) described the BHA-mediated induction of a class alpha mRNA that encoded a protein sharing 95% homology with the rat Ya subunit. The rat cDNA clone was used as a probe for this species. Thus, the rat Ya clone used in these experiments to probe mouse hepatic RNA most probably hybridises to the Ya_1 subunit rather than Ya_3. The pattern of expression at the mRNA level reflects that seen at the protein when mouse hepatic cytosol is immunoblotted against anti-mouse Ya_1 antisera. This lends further weight to this assumption.

5.04 Effect of xenobiotic inducing agents on the hepatic GST profile of little mice

5.04a Animal treatments

Male and female little mice (lit/lit) plus heterozygote (lit/+) and wild-type controls were obtained from Charles Rivers laboratories (Willmington, USA) at the

Figure 5.14

Northern blot analysis using the rat Yf GST cDNA clone of sham operated and hypophysectomised animals treated with xenobiotics.

Hepatic GST mRNA levels were invesigated by northern blot analysis as described in section 2.12. Liver RNA (15μg) was electrophoretically size-fractionated using a 1% (w/v) agarose gel with 2.2M-formaldehyde. After electrophoresis, the RNA was transferred to nitrocellulose and hydridised at 65oC overnihgt with the Yf cDNA clone. Loadings were as follows :-

Panels a and b. lane 1, sham male; lane 2, sham female; lane 3, hypox male; lane 4, hypox female; lane 5, sham male treated with PB; lane 6, sham female treated with PB; lane 7, hypox male treated with PB; lane 8, hypox female treated with PB; lane 9, sham male treated with DEX; lane 10, sham female treated with DEX; lane 11, hypox male treated with DEX; lane 12, hypox female treated with DEX; lane 13, sham male treated with TCBOP; lane 14, sham female treated with TCBOP; lane 15, hypox male treated with TCBOP; lane 16, hypox female treated with TCBOP.

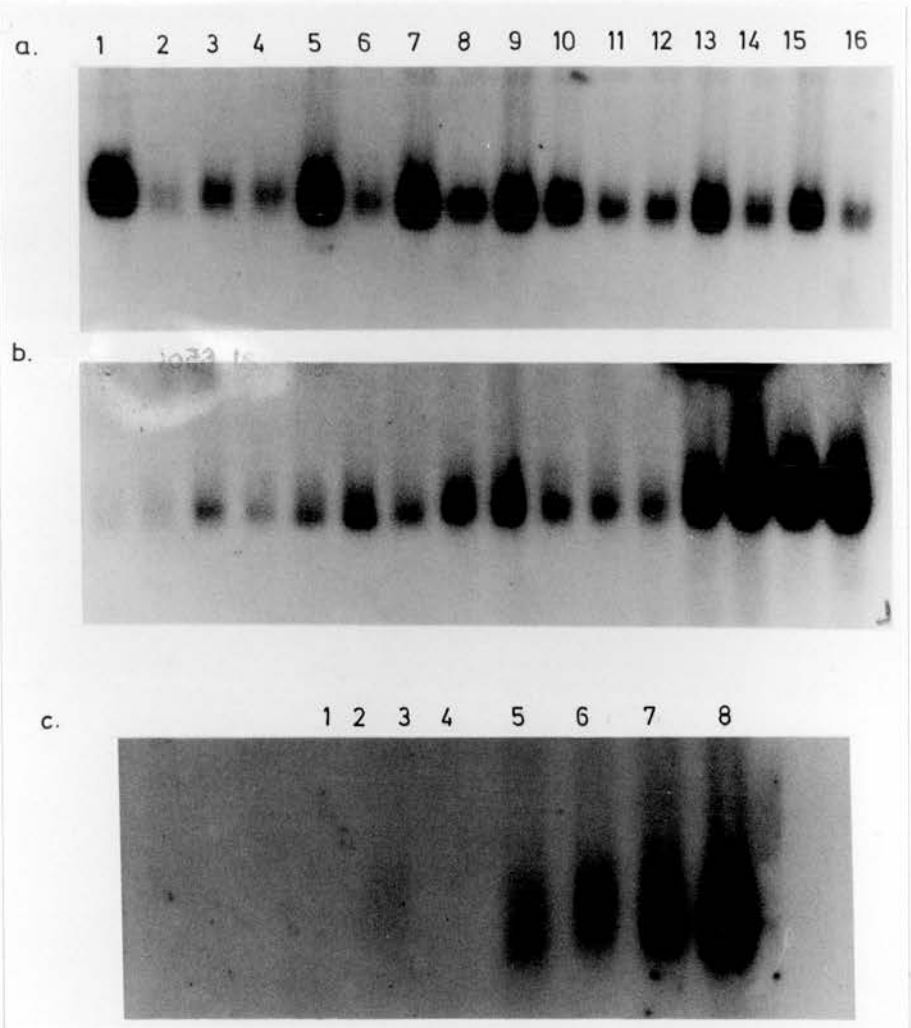
Panel c. lane 1, sham male; lane 2, sham female; lane 3, hypox male; lane 4 hypox female; lane 5, sham male treated with TCBOP; lane 6, sham female treated with TCBOP; lane 7, hypox male treated with TCBOP; lane 8, hypox female treated with TCBOP.

Panel a. probe - the rat Yf cDNA.

Panel b. probe - the rat Yb_1 cDNA.

Panel c. probe - the rat Ya cDNA.

Figure 5.14



age of 8 weeks. After transportation the mice were acclimatized for 2 weeks before the commencement of treatment. Phenobarbital and dexamethasone were administered using an identical treatment regime to that undertaken for the experiments described in sections 5.02a, 5.03a, i.e. PB was injected interperitoneally (ip) at a dose of 80mg/kg/day for 3 days and Dex was administered (ip) at 100mg/kg/day for 4 days. Controls received corn oil. A summary of the treatment groups and the numbers per group is given in table 5.02

5.04b Analysis of hepatic GST content.

Phenobarbital and dexamethasone were found to effect the hepatic CDNB activity in the little mice in a similar manner to that observed for wild-type C57BL/6 mice in previous experiments (figure 5.15). For some reason, treatment of the wild-type did not lead to an increase in CDNB activity, although Yb levels were induced (figure 5.17) These compounds were found to cause an increase in the level of Yb-type subunits in the livers of both male and female little mice. The constitutively expressed Ya_3 subunit was unaffected (figures 5.16, 5.17)

As has been previously discussed, little mice were found to exhibit a feminine pattern of GST expression, the Yf being expressed at female levels. A slight increase in the Yf was observed in the little males treated with PB. It must be emphasized that the results presented are of a preliminary nature, further work would have to be undertaken to confirm this finding.

Immunoblotting does not discriminate between different members of the Yb gene family. It is not known which Yb subunit(s) are actually being induced after treatment with PB and Dex. The elevation in DCNB activity observed on treatment with xenobiotics provides strong evidence that the Yb_1 subunit is induced, this does

not exclude the possibility that other members of this family (i.e. Yb_2 and Yb_3) are also regulated by this treatment (figure 5.18).

Table 5.02 Response of little mice to xenobiotics - a summary of treatment experimental treatment groups

Phenotype	Sex	Treatment	Animal No.
Lit/Lit	male	corn oil	3
	female	corn oil	3
Lit/+	male	corn oil	3
	female	corn oil	3
+/+	male	corn oil	3
	female	corn oil	3
Lit/Lit	male	PB	3
	female	PB	3
Lit/+	male	PB	3
	female	PB	3
+/+	male	PB	3
	female	PB	3
Lit/Lit	male	DEX	4
	female	DEX	4
Lit/+	male	DEX	4
	female	DEX	4
+/+	male	DEX	4
	female	DEX	4

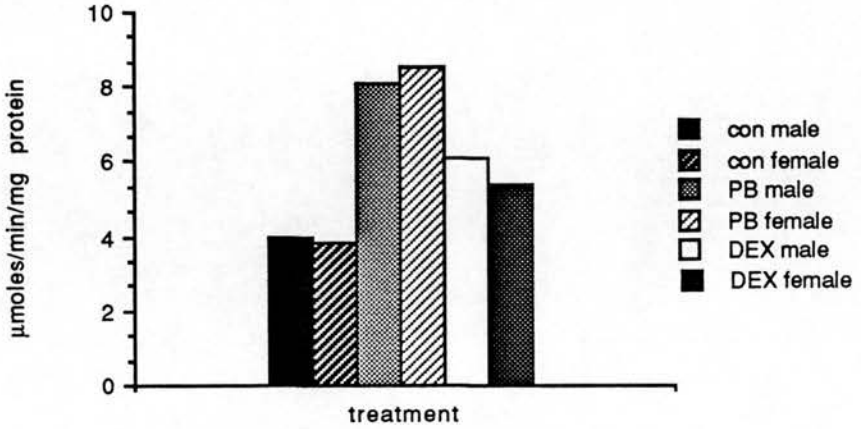
Abbreviations: PB - phenobarbital; DEX- dexamethasone.

Figure 5.15

Levels of CDNB activity in little mice and wild-type C57BL/6 mice treated with xenobiotic.

Figure 5.15

a. CDNB activity - little mice



b. CDNB activity - wild-type C57BL/6 mice

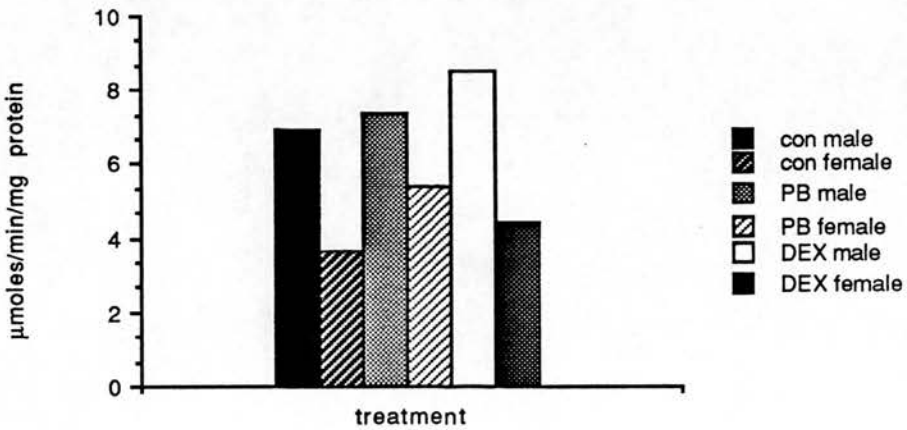


Figure 5.16

Electrophoretic analysis of hepatic cytosols from little and wild-type mice treated with xenobiotics.

Pooled cytosols (30μg) were analysed by SDS/PAGE on a 12% (w/v) polyacrylamide resolving gel. Loadings were as follows :- lanes 1,8,15, rat liver standards (Yc,Yb,Ya); lane 2, lit/lit male control; lane 3; lit/lit female control; lane 4, lit/lit male treated with PB; lane 5, lit/lit female treated with PB; lane 6, lit/lit male treated with DEX; lane 7, lit/lit female treated with DEX; lane 9, +/+ male control; lane 10, +/+ female control; lane 11, +/+ treated with PB; lane 12, +/+ female treated with PB; lane 13, +/+ male treated with DEX; lane 14, +/+ female treated with DEX.

Figure 5.16

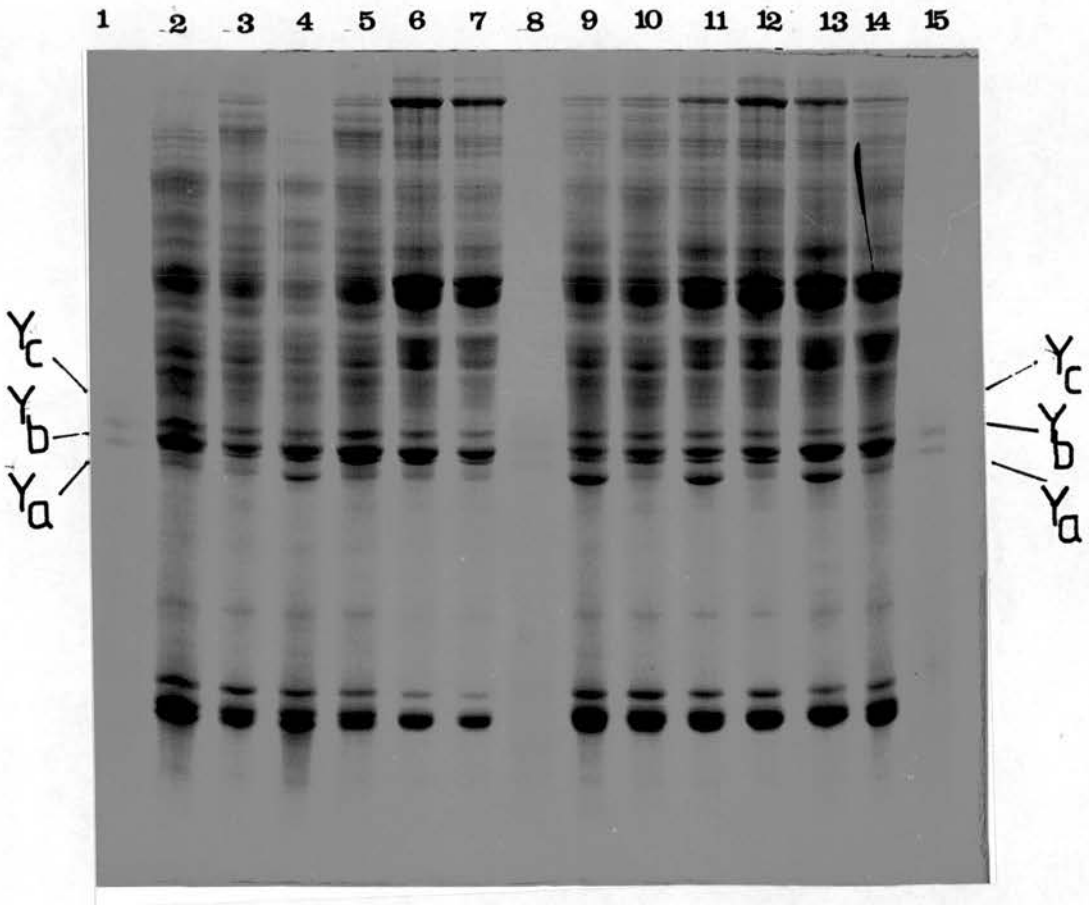


Figure 5.17

Immunoblotting of hepatic cytosols from little and wild-type mice treated with xenobiotics.

Pooled cytosols (50μg) were subject to SDS/PAGE, followed by transfer to nitrocellulose and immunoblotted with anti-mouse GST antisera. The gel was loaded as follows :- lanes1 and 8, GST markers; lane2, male control; lane 3, female control; lane 4, male treated with PB; lane 5 female treated with PB; lane 6, male treated with DEX; lane 7, female treated with DEX.

Panel a. lit/lit mice

Panel b. +/+ mice

Figure 5.17

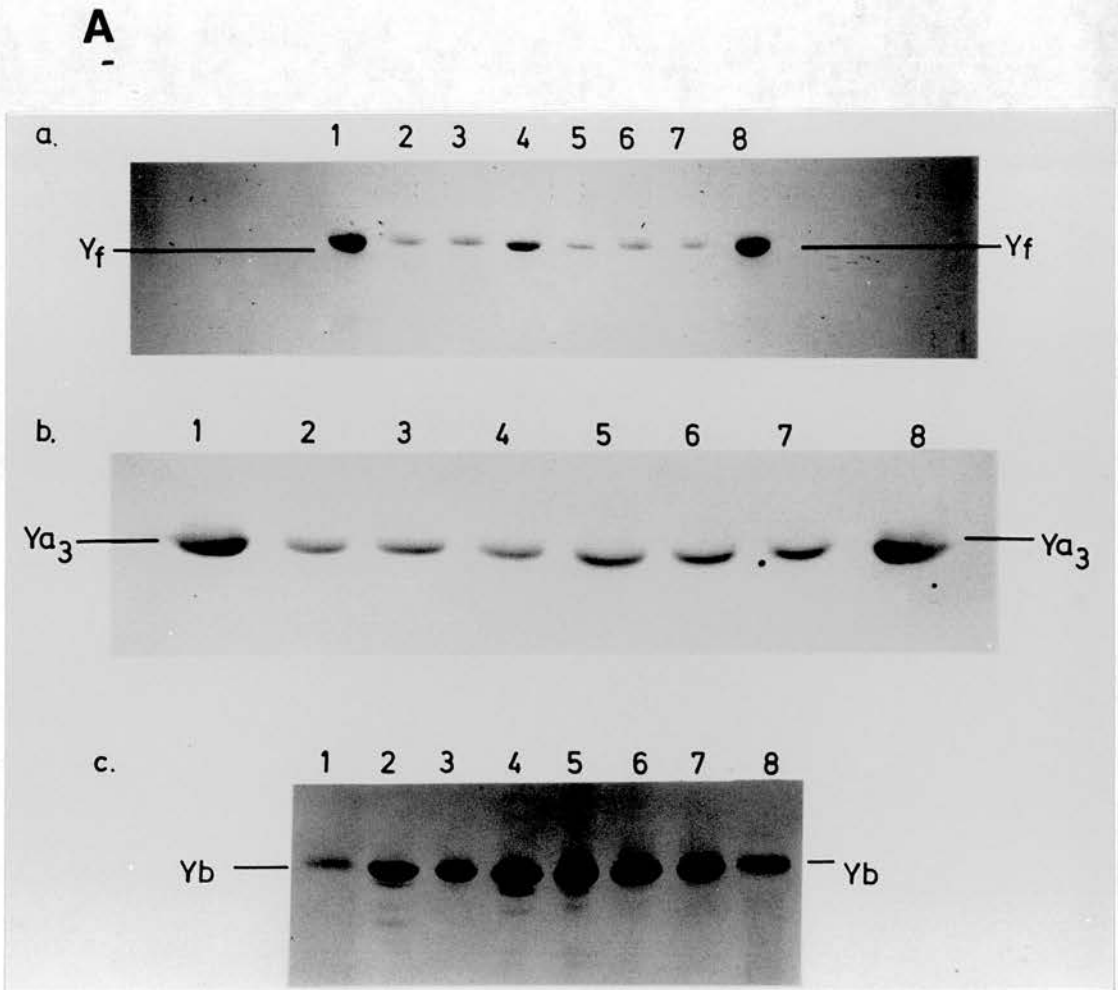


Figure 5.17

Immunoblotting of hepatic cytosols from little and wild-type mice treated with xenobiotics.

Pooles cytosols (50μg) were subject to SDS/PAGE, followed by transfer to nitrocellulose and immunoblotted with anti-mouse GST antisera. The gel was loaded as follows :- lanes1 and 8, GST markers; lane2, male control; lane 3, female control; lane 4, male treated with PB; lane 5 female treated with PB; lane 6, male treated with DEX; lane 7, female treated with DEX.

Panel a. lit/lit mice

Panel b. +/+ mice

Figure 5.17

B

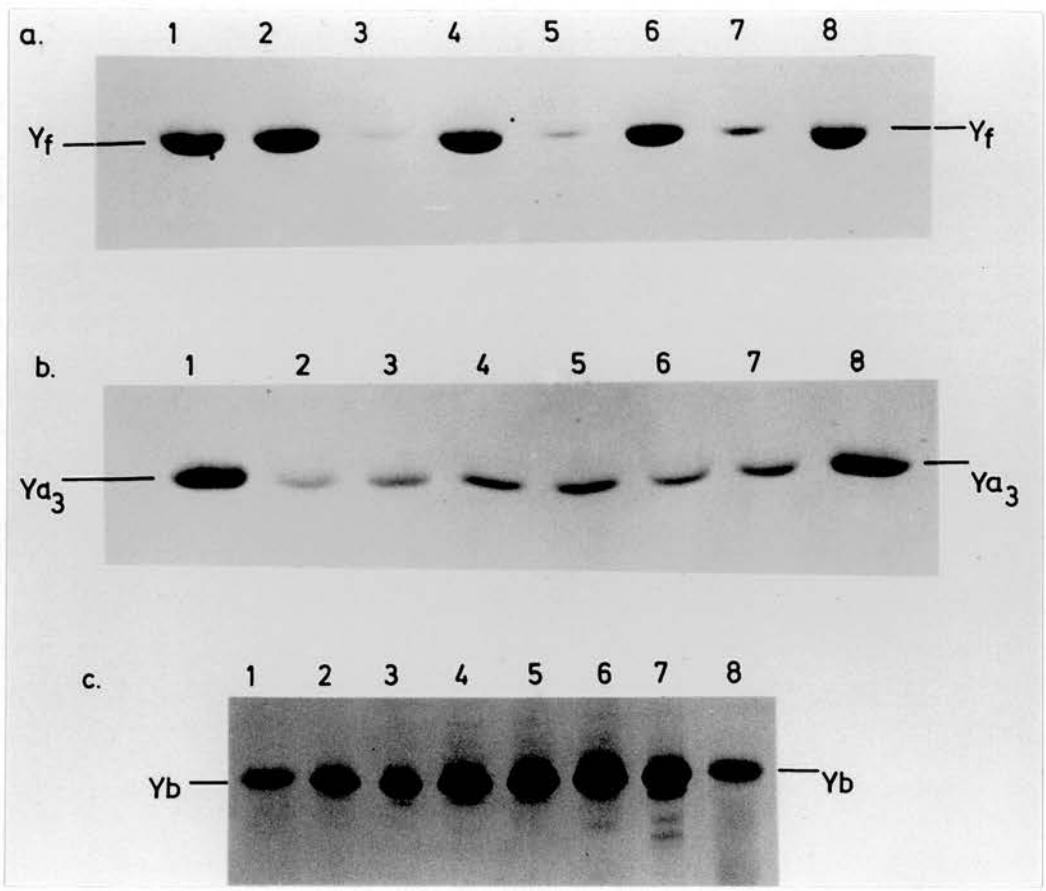
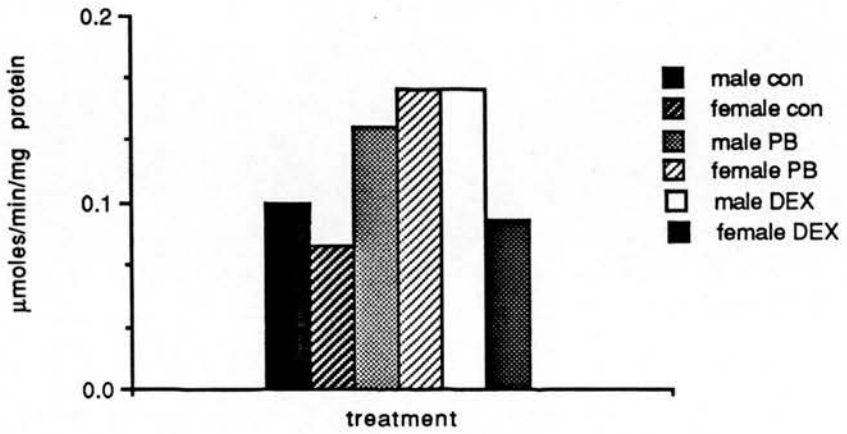


Figure 5.18

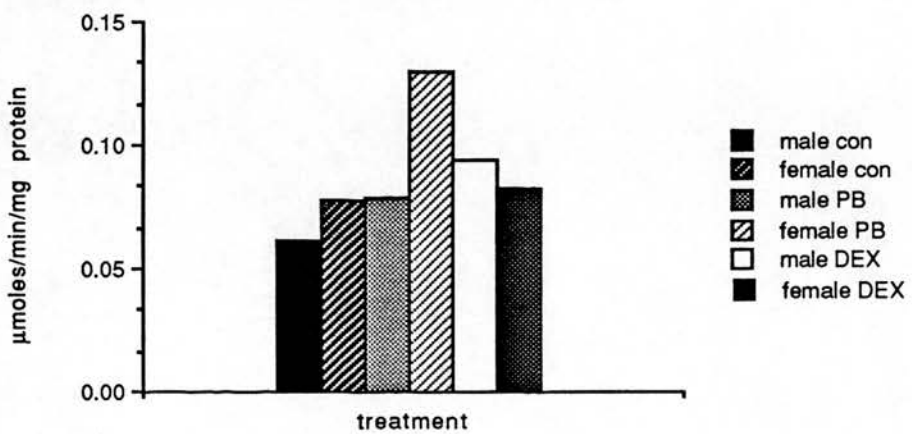
Levels of DCNB activity in little mice and wild-type C57BL/6 mice treated with xenobiotic.

Figure 5.18

a. DCNB activity - little mice



b. DCNB activity - wild-type C57BL/6 mice



5.05 Discussion

5.05a Summary of results

The effect of phenobarbital and the 'phenobarbital-like inducers', dexamethasone and TCBOP on mouse liver hepatic GST content of both sexes was investigated in two strains of mice, C57BL/6 and DBA/2. Preliminary results indicate that xenobiotic treatment resulted in an induction of the Yb gene family. No sex differences in induction were apparent. Interestingly though, in the DBA/2 strain DCNB activity was consistently lower in the female. On western blotting however with anti-mouse Yb_1 antisera, no notable differences were detected. The Ya_3 and Yf subunits did not appear to be significantly affected by the administration of these compounds. The increase in DCNB activity is in good accord with the findings of David and Nerland (1983) who reported DCNB activity was elevated approximately 5-fold after male mice were placed on a liquid diet of 0.5mg/ml phenobarbital for 7 days. Di Simplico *et al.*, (1989) have also reported the mu class GST to be induced after phenobarbital treatment. Interestingly, a greater effect was observed when mice were administered phenobarbital as part of a their diet as compared to interperitoneal injection.

Phenobarbital and dexamethasone induced hepatic GST to a similar level, the induction being more pronounced in the C57BL/6 strain. TCBOP was found to be the most potent compound of the three tested, CDNB activity was elevated approximately 2-fold after TCBOP treatment, while DCNB activity was increased approximately 5-fold. Maximum induction of the Yb family with TCBOP occured 2-4 weeks after a single injection of TCBOP at 3mg/kg. Expression gradually fell back towards control levels over a 8-10 week period. This occurs in both strains and sexes, again the C57BL/6 mice exhibited a greater response to the compound. Even at the 12 week

time-point, DCNB activity had not completely fallen to control values. This prolonged response to TCBOP is also observed for the cytochrome P-450 family and probably reflects the biological half-life of this compound which has been shown to persist for up to 30 days after administration (Poland *et al.*, 1980). Differential sensitivity in the induction of PB inducible P-450 genes also occurs between these strains although in the opposite orientation. Induction of the coumarin hydroxylase locus by phenobarbital is greater in the DBA/2 strain than in the C57BL/6 strain (Wood and Taylor,1979).

5.05b Mechanism of action

Many factors could influence the differential sensitivity observed between the strains, for example, differences in the metabolism of these compounds, differences in receptor concentration or affinity towards these compounds. The mechanism of phenobarbital induction is not yet understood. No receptor has as yet been identified. It is not known whether the effects of PB and TCBOP treatment are mediated through the same pathway, but in the case TCBOP there is some evidence to suggest that a receptor might be involved (Kende *et al.*,1985; Kelley *et al.*, 1985) Whether this putative receptor does exist and is related to the hypothetical PB receptor has as yet to be established.

The pathway by which polycyclic aromatic hydrocarbons (PAH) regulate the expression of drug metabolising enzymes has been the best documented system to date. A high affinity cytosolic receptor, the product of the Ah locus, binds to the PAH inducer. This ligand receptor complex is translocated into the nucleus where its interaction with DNA results in the transcription of induction-specific mRNAs. This will be discussed in more detail in chapter 6 (section 6.07). Certain strains of mice

including the DBA/2 strain lack the Ah receptor. By contrast, C57BL/6 mice are positive for this receptor. Both strains were affected by the xenobiotics administered. This is not surprising as induction of PB-inducible genes occurs by a separate pathway.

5.05c Dexamethasone : only a synthetic glucocorticoid?

Hormones of the adrenal are subdivided into two distinct groups. a) The glucocorticoids which affect fat, protein and carbohydrate metabolism as well as regulating the immune system. b) The mineralcorticoids which control the sodium/ potassium balance. Glucocorticoid actions are mediated via the glucocorticoid receptor. Dexamethasone is a synthetic steroid with potent glucocorticoid activity which has been used to simulate the action of natural glucocorticoids. An increasing body of evidence emphasizes the point that care must be taken when interpreting effects observed after dexamethasone treatment.

Tredger *et al.* (1976) showed that natural glucocorticoids, which are normally rapidly metabolised, had the opposite effect to the potent long-lasting synthetic compounds, which are slowly metabolised. Natural glucocorticoids were found to suppress biphenyl hydroxlylase and ethylmorphine N-demethylase activity and lead to a decrease in cytochrome P-450 levels in rat liver. In contrast, dexamethasone increased these parameters by approximately 2-fold. In the rat, dexamethasone is a good inducer of a major member the of the PCN P-450 family, in fact dexamethasone was found to be a more potent inducer than PCN itself. Natural glucocorticoids were however very poor inducers of this enzyme. The authors concluded that the induction noted was not mediated via the glucocorticoid receptor (Schuetz and Guzelian, 1984).

The fact that dexamethasone acts in a similar way to phenobarbital and TCBOP would suggest that dexamethasone is not acting via the glucocorticoid receptor, although this possibility cannot be ruled out. Unfortunately to date no data have been presented on the effect of adrenalectomy or replacement therapy with natural glucocorticoids on hepatic GST content in either the rat or mouse.

Interestingly, the Yb subunit in the rat has been found to bind dexamethasone-21-methanesulphonate which is an effective affinity label for glucocorticoid binding proteins (Homma and Listowsky, 1985). The Yb subunit has also been isolated from rat nucleus complexed to DNA (Bennet *et al.*, 1986). There may be a possibility that GST might also play a role in the intracellular transport of hormones, acting in this case as binding proteins. It is difficult to establish whether the presence of GST in the nucleus is a genuine phenomenon or whether it is simply an artefact of the preparation

5.05d The involvement of the pituitary gland in the induction of mouse hepatic GST.

Hypophysectomy in the rat has been found to enhance the induction of certain members of the P-450 family inducible by phenobarbital. Removal of the pituitary has been found to increase the basal level of expression of these P-450 genes, suggesting that certain factors in the pituitary normally repress their expression. This increase in base-line level is further enhanced by treatment with PB-type xenobiotics. Evidence presented suggests that the pituitary factor responsible for this suppression is growth hormone (Yamazoe *et al.*, 1987).

The induction of the mu class family was not significantly affected by hypophysectomy, this indicates that the pituitary is not involved in influencing the

induction of this class by xenobiotics. Further confirmation that growth hormone or its absence does not affect the induction of hepatic GST by xenobiotic compounds is shown in section 5.04 where little mice of both sexes were found to respond to the administration of xenobiotics in a normal fashion.

However in the case of the Ya_1 alpha class subunit, hypophysectomy leads to an increase in the expression of this subunit in both male and female C57BL/6 mice. This has been demonstrated previously, little mice are found to have increased levels of this subunit over that found in the heterozygote and wild-type controls (see section 4.03.). It would therefore appear that the Ya_1 subunit is under repressive control control of growth hormone. TCBOP treatment also induces the level of this subunit. With both treatments combined, an additive effect on the induction of this alpha class member is noted, particularly in the female.

No sex differences in the induction of hepatic GST was readily apparent in either control or hypophysectomised animals in regard to the Yb-class GST. This is in contrast to the rat where PB and 3-MC induce hepatic GST in a sex-specific manner (Igarashi *et al.*, 1987). PB treatment was found to induce CDNB activity by a greater fold induction in the adult male as compared to the female, thereby increasing the differences between the sexes. Treatment with 3-MC elicited a greater response in the female rat, lessening the difference between the sexes. A similar situation occurs for other xenobiotic-inducible enzymes which are sexually differentiated (see Skett, 1987). Thus, sex-specific effects noted in foreign compound induction seem to occur when the proteins themselves are also sexually differentiated.

5.05e Further work

These data are of a preliminary nature, a more detailed study of TCBOP and its effects on hepatic GST content would be very interesting, with particular attention to the mu class GST. Individual Yb-type subunits could be isolated and purified by reverse-phase h.p.l.c. after treatment with TCBOP. Evidence suggests that the Yb_1 subunit is induced after administration of this compound but it is possible that other members of the family are also more highly expressed.

In respect to the induction of P-450 genes by TCBOP in the mouse, this compound is estimated to be 650-fold more potent than phenobarbital in its effect. In the rat however, administration of TCBOP at a similar concentration to that given in the mouse has no effect on P-450 levels. Its thought that a species difference in the presence of the receptor for TCBOP is the reason which belies this finding (Poland *et al.*, 1981). With this hypothesis in mind, the prediction would be that TCBOP would not affect the induction of hepatic GST in the rat. Furthermore, it would suggest that phenobarbital and TCBOP, although having similar affects with different intensities between species, do not act by an identical route. This work presented within this thesis has concentrated exclusively on the liver, however, TCBOP has also been shown to affect other tissues most notably the intestine and also the kidney (Poland *et al.*, 1980). Very little data is available on the effects of xenobiotics in extrahepatic tissue. An investigation into the effects of the long-lasting TCBOP on tissues other than the liver would be worthwhile.

Only a brief discussion on the effects of foreign compounds on hepatic GST content has been given in this section. A fuller account and an in-depth discussion of the

molecular mechanism by which these compounds exert their effect is given in chapter 6 section 6.08.

Chapter 6 - Discussion

6.01 Sex-specific regulation of mouse hepatic YfYf

The major aim of the work presented within this thesis was to investigate the mechanism which gives rise to the marked sexual dimorphism in the expression of the mouse hepatic Yf subunit. The evidence presented strongly suggests that the male sex hormone testosterone regulates the Yf GST through an indirect mechanism. Androgenic effects on the Yf GST are mediated via growth hormone or more specifically, through the growth hormone secretory pattern. Figure 6.01 illustrates the proposed mechanism.

In the male, soon after birth, a brief surge in testosterone production in some manner, as yet not understood, pre-programmes the hypothalamus such that the pituitary will secrete growth hormone in a pulsatile fashion when testosterone is present. Testosterone, produced at the onset of puberty by the Leydig cells of the testes, acts on this "pre-set" hypothalamus which in turn stimulates the pituitary to produce growth hormone in a sex-specific manner.

Two factors are produced by the hypothalamus which control the release of growth hormone from the pituitary somatotrophs. Somatostatin exerts a negative effect on growth hormone production, and growth hormone-releasing factor (GHRF), which acts positively on the pituitary to stimulate the synthesis and secretion of growth hormone. The finer details of this pathway are not completely understood yet but the evidence suggests that testosterone in some manner regulates the production of hypothalamic somatostatin to produce the sex-specific rhythm of growth hormone

secretion (see Zaphiropoulos *et al.*, 1989). The male-specific secretory mode in some manner signals the expression of male-specific proteins in the liver.

Interference in this pathway, either through mutation (as in the mouse model) or through inactivation of particular parts of the pathway (by for example surgery,) leads to a feminine pattern of gene expression. Marked on the diagram (figure 6.01) are the disruptions to this pathway which occur in the various models which have been investigated during the course of this work.

How growth hormone binding to its receptor on the surface of the liver actually regulates expression of the hepatic YfYf and other sexually dimorphic liver proteins is not known. The proposed mechanism by Norstedt and Palmiter (1984), presented and discussed in section 4.05c uses the dichotomy of growth hormone action i.e. its ability to act directly through its receptor or indirectly through the somatomedins, to fulfil its dual function in regulating both male and female specific expression. Obviously this represents a key question in this area which should be addressed over the next few years.

6.02 Why is the Yf subunit subject to sexual dimorphism in mouse liver?

The reason behind the overexpression of Yf in male mouse liver has not yet been uncovered but the difference in the levels of this polypeptide between the sexes is very marked. Expression of the Yf in the male mouse is approximately 10-fold higher than in the female and two orders of magnitude higher than seen in the rat liver of either sex (Hatayama *et al* ., 1986).

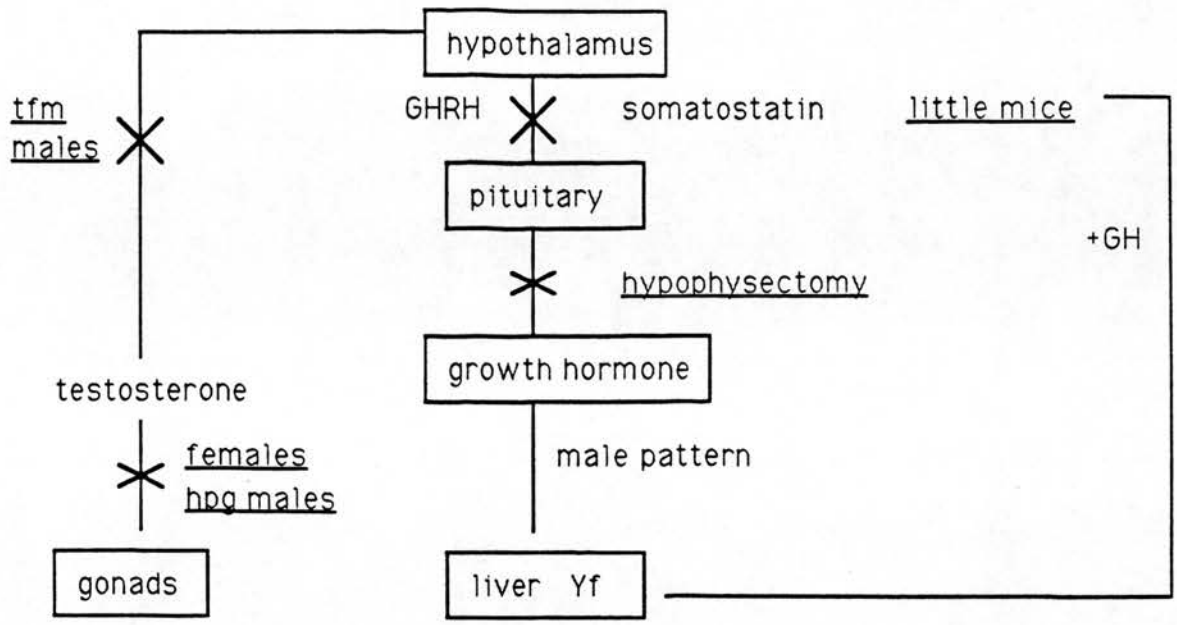


Figure 6.01

Scheme for the regulation of mouse hepatic GST YfYf levels.

In the male, a brief surge of testosterone in the neonatal period in some manner pre-programmes the hypothalamus in a male-specific fashion. At the onset of puberty, testosterone in some manner stimulates the male-specific pattern of growth hormone secretion. It is believed that testosterone controls the release of the hypothalamic factor somatostatin which acts to inhibit growth hormone release from the pituitary. The male-specific pattern of growth hormone release, by some pathway as yet unidentified, acts to regulate the expression of the Yf subunit in mouse liver. Marked on the diagram are the disruptions to this pathway in the various models studied during the course of this work, which result in a female level of YfYf expression.

As regards other sexually differentiated hepatic proteins, it is thought that the major purpose behind this dichotomy is the maintenance of the proper hormonal balance and the ability to respond to these hormones. This obviously differs between the sexes. It is conceivable that GST YfYf is also involved in maintaining a male-specific metabolic pathway, acting on a physiological substrate as yet unidentified. Glutathione S-transferases, in addition to their GSH conjugating activity have also been shown to have isomerase activity, catalysing the conversion of Δ^5-3-oxo-steroids to the corresponding Δ^4-isomers (Benson *et al.*,1977). Steroids have been reported to interact with the human pi class GST in vitro both as substrates and inhibitors. However, the kinetics of the reaction would indicate that these interactions are of minor physiological significance (Dibbelt *et al.*, 1988). This finding suggests steroids are unlikely candidates for the potentially sex-specific substrate for the mouse Yf subunit. However due to the possibility of species differences, this cannot be entirely dismissed.

Until recently, the pi class GST family was thought to contain a single member, the product of one gene (Okuda *et al.*, 1987). A recent finding challenges this assumption. Fatty acid ethyl esters (FAEE) are the end products arising from the nonoxidative metabolism of alcohol. These products are synthesized by two synthetase enzymes . The major synthetase (pI 4.9) has recently been purified from human heart muscle. The N-terminal 23 amino acids have been sequenced and found to be 73% identical to the rat Yf GST and 82% identical to the N-terminal sequence of the human Yf GST purified from human heart. Catalytic activities were interchangeable, in that FAEE synthetase was found active in catalysing the conjugation of CDNB and GSH, while a homogeneous GST isolated from bovine liver (Mr 26,000) was active in the formation of FAEE (Bora *et al.*, 1989)..

The Yf has also been shown to be reactive with lipids. Ketterer and his co-workers demonstrated that form 7-7 (YfYf) purified from primary rat hepatoma had Se-independent GSH activity towards lipid peroxides (Meyer *et al.*, 1985). GST P (YfYf) was found by Sato to have significant activity towards 4-hydroxynonenal, a potent lipid peroxide, although the rat Yk subunit has highest activity (Sato,1988, Danielson *et al.*, 1987). Tsuchida *et al.* (1987) reported that GST form 7-7 also has significant leukotriene C4 synthase activity, although again a mu class GST purified from rat brain (Yn_1Yn_1) has greatest activity.

In rat, no physiological substrate for the Yf GST has been identified. Although, as detailed above, it would appear to interact with long chain fatty acid compounds, demonstrating diverse catalytic activities. Bearing in mind the sex-specific expression of Yf in the mouse and its as yet hypothetical role in a male-specific process of some sort, possible physiological candidates might be the prostaglandins or leukotrienes. These are signal- type molecules derived from arachidonic acid. The prostaglandins, of which there are nine distinct classes, are involved in a wide variety of physiological processes and would appear to modulate the action of hormones rather than act as hormones themselves. Thus, Yf might be involved in the formation of a specific class of these signaling molecules and in this way play a role in a male-specific process. It must be emphasized that this is purely hypothetical, but it would certainly be of interest to determine if the mouse Yf GST had any catalytic activity to the aforementioned putative substrates.

An analysis of the 5' flanking region of the human genomic Yf sequence (Cowell *et al.*,1988) reveals a region from position -100 to +300 where the G+C content reaches 72% and the proportion of CpG dinucleotides is 9.2%. This description is characteristic of HTF islands (Hpa II tiny fragments). These regions are

constitutively hypomethylated and are particularly associated with house-keeping genes, which are intrinsic for the maintenance of all cell-types. Hypomethylation is a feature associated with genes which are under active transcription. It may be that Yf has a fundamental biological role in at least certain cell-types. Findings described below, linking the expression of the rat hepatic Yf GST with that of genes involved in cellular growth and division, suggest that in the rat hepatocyte the Yf subunit might have a role to play in preneoplastic proliferation. If that is the case in the mouse, then it might have profound biological consequences for the animal. This will be discussed in section 6.03.

6.03 Regulation of hepatic GST in the rat

6.03a Expression of the Yf subunit in altered hepatocyte nodules

With the exception of the mouse, all other rodent species studied so far including the rat are found to have only trace amounts of the Yf subunit in the liver (Hayes *et al.*, 1987). By contrast, extrahepatic rat tissue, including the kidney, lung, spleen, thymus and small intestine, contain significant amounts of this GST. Rat testes, like the liver were found to exhibit extremely low levels of this subunit (Hayes and Mantle, 1986a). No sex difference in the hepatic level of the Yf subunit has been reported in the rat. Due to the low constitutive expression of this subunit in this species, any slight differences might go unnoticed.

Administration of a single dose of diethlynitrosamine (DEN) followed by three consecutive doses of 2-acetlyaminofluorene (2-AAF) plus a partial hepatectomy induces foci of altered hepatocytes referred to as preneoplastic nodules (also

persistent / hyperplastic nodules). This model is used to study the stages involved in chemically -induced hepatocellular carcinoma and is referred to as the Solt-Farber model or the resistant hepatocyte model (Solt and Farber, 1976). As the term suggests, preneoplastic nodules are thought to serve as precursors in the generation of liver cancer.

Changes in the glutathione S-transferases and other drug-metabolising enzymes occur during the formation of preneoplastic nodules. In general, Phase I drug-metabolising enzymes are depressed whereas Phase II enzymes are elevated. In addition to GST, the levels of DT-diaphorase and epoxide hydrolase are found to be increased in these altered hepatocytes (Pickett *et al.*, 1987). As regards individual GST subunits, all except Yb_2 (which is depressed) are elevated in nodule-bearing livers. The Yf GST is dramatically increased during this precancerous stage to levels between 10 and 100-fold higher than found in normal rat liver (Satoh *et al* 1985). The derepression and /or induction of the Yf is a rapid event occurring soon after the initiation of the process. On immumohistochemistry, small foci of hepatocytes are found to stain heavily with antisera raised against YfYf 48 hours after a single initiating dose with hepatocarcinogen (Moore *et al.*, 1987). Elevation in other preneoplastic markers such as γ-glutamlytranspeptidase were not apparent at this stage. A similar sequence of events in respect to Yf is observed using models other than the Solt-Farber to induce neoplasia. (Eriksson *et al.*, 1983; Roomi *et al.*, 1983). Considerable attention has therefore focussed on the Yf as a possible marker for preneoplasia.

Unlike other drug-metabolising enzymes, GST YfYf is not affected by xenobiotic compounds such as phenobarbital and 3-methylcholanthrene (Roomi *et al.*, 1982; Satoh *et al.*, 1985). However lead nitrate, which induces hepatocyte cell

proliferation has been found to cause an elevation in Yf GST levels only at a dose which stimulates cell proliferation (Roomi *et al.*, 1987). Other changes occur in lead nitrate treated livers which are similar to those exhibited during preneoplasia. It has been be noted that partial hepatectomy in the rat does not induce YfYf (Power *et al.*, 1987). This would suggest that an increase in the levels of YfYf is not a normal feature of cellular growth and proliferation. It is possible therefore that the Yf is being induced after lead nitrate administration as part of the preneoplastic-type proliferative response rather than a direct effect of the compound, although this possibility has not been ruled out. Induction of cell proliferation by lead nitrate might in future serve as a useful model in which to study those changes commonly associated with hepatocellular carcinoma.

The role of GST YfYf in the development of neoplastic foci in the liver has yet to be clarified. The induction of this enzyme during the early initiation stages in all models tested presents the possibility that Yf might have a fundamental function in this process, perhaps playing a role in the initiation of altered growth in the hepatocyte. Recent findings linking the expression of the Yf GST to genes involved in the control of cellular growth lends credence to this supposition. This will be discussed in detail in section 6.03b. Other functions have also been proposed which are briefly discussed below.

Altered hepatocyte foci are resistant to the effects of cytotoxic agents (Farber 1984 a,b). In view of the changes observed in the drug-metabolising enzymes of both classes, it is not unduly surprising that preneoplastic cells exhibit less susceptibility than normal cells to toxic chemicals. It may be that the Yf GST subunit is involved in the generation of this resistant phenotype, although it should also be noted that other GST forms are also induced which probably contribute to the

resistant phenotype. The Yf GST has been reported by Ketterer and co-workers as having Se-independent peroxidase activity towards lipid peroxides (Meyer *et al.*, 1985). Sato and his colleagues found this subunit to have high activity towards 4-hydroxynonenal, a potent lipid peroxide derived from arachidonic acid (Sato, 1988). In view of these findings, Sato has speculated that the induction of the Yf GST may be related to the prevention of lipid peroxidation which has an important role to play in tumour promotion (Sato, 1988).

Interestingly, YfYf has been detected in the nucleus by immunohistochemistry. Preneoplastic hepatocytes, preneoplastic foci from rat hamster pancreas and human cervix dysplatic cells have been shown to contain Yf in the nucleus. Normal hepatocytes from the normal male mouse also show nuclear staining. Other GST subunits were not detected in the nucleus (Sato, 1988). This is in contrast to Bennet *et al.* (1986), who found the Yb_1 subunit complexed to nuclear hepatic DNA, although this was in normal rat liver. The significance of these findings are not clear. It may be that the Yf serves as a carrier ligand for some compound as yet unidentified. Alternatively, as part of a general protective function, GST YfYf might form a "final barrier" of drug-detoxification enzymes attempting to prevent reactive electrophiles from interacting with the DNA. Obviously, further work will have to be undertaken to confirm these findings and to investigate the function of GST YfYf found within a nuclear location.

6.03b Oncogene mediated expression of the rat hepatic Yf GST.

The mechanism underlying the dramatic derepression of the Yf GST subunit during preneoplasia has been the subject of considerable interest. A genomic sequence for the rat Yf GST was obtained by Okuda *et al.* (1987). Multiple regulatory elements

have been described which are located 5' to the coding region. Cis-acting elements were analysed by fusing various lengths of the 5' flanking sequence to the bacterial chloramphenicol transacetylase gene and transfecting these constructs into a rat hepatoma cell-line (Sakai *et al.*, 1988).

Two enhancing elements have been identified 2.5kb and 61bp upstream from the transcription start site. The upstream enhancer was divided into two domains GPE1 and GPE2. The GPE 1 domain and the downstream enhancer sequence (at 61bp) contain a 12-O-tetradecanoylphorbol-3-acetate (TPA) responsive element (TRE). A silencer region was also described 400bp upstream from the cap site which may be important in the repression of Yf activity in the normal rat liver. Moreover, the endogenously expressed Yf GST in rat fibroblast 371 cells was also responsive to TPA.

Phorbol esters such as TPA are potent tumour promoters, capable of potentiating the effect of subcarcinogenic doses of an initiating carcinogen. Among the genes transcriptionally activated by TPA are the oncogenes c-myc and c-fos (Angel *et al.*, 1989). It has been suggested that the alterations in gene expression following TPA administration involve a signal transduction cascade, triggered by protein kinase C. The human pi gene has also been found to contain TRE sequences in the promoter region (Cowell *et al.*, 1988)

The oncogene c-Ha-ras has also been shown to act through TRE-like sequences (Imler *et al.* 1988). This could be highly significant in view of the the fact that many human tumours are ras-related and GST has been reported also being induced in a variety of tumour types (Shea and Henner, 1987).

Recently, it has been shown by two independent groups, that transfection of the ras gene into cultured cells results in the induction of the Yf GST together with other genes involved in hepatocarcinogenesis or the drug-resistant phenotype. Burt *et al.*(1988) transfected the viral oncogenes v-Ha-ras and v-raf into a rat liver epithelial cell-line (RLE). A marked induction of the Yf GST was observed in the oncogene transfected cells. P-glycoprotein, a 170,000 ATPase-dependent membrane transport protein believed to actively pump cytotoxic agents out of the cell, was also elevated in these cells. Transfected cell lines were found to have enhanced resistance to the effects of the anticancer drugs, vinblastine and adriamycin, as well as the hepatocarcinogen, 2-acetylaminofluorene.

Li *et al.*, (1988) reported similar findings. The cellular ras gene linked to a metallothionein promoter, inducible with zinc, was transfected into rat liver epithelia cells. Again, elevation in the levels of the Yf GST was observed and also in γ-glutamlytranspeptidase (γ-GT), another GSH-dependent enzyme elevated during chemically-initiated carcinogenesis of the liver.

Data demonstrating the inducibility of the Yf with tumour promoters and genes linked to cellular growth and division, could explain the increase in Yf noted in preneoplasia and in many tumour types. Activated ras genes do not appear to be a common feature of carcinogenic lesions (Reynolds *et al.*, 1986), which suggests that induction of YfYf might be a common feature of the initiation of transformation by several mechanisms. Power *et al.* (1987) have concluded that elevation of Yf is particularly associated with the initiation of the transformed phenotype and does not have a role to play in either cell proliferation or protection against toxicity.

In conclusion, the question still remains as to the function of this protein (if any) during the carcinogenic process. From the findings of various laboratories, which have been discussed above, it would appear that the Yf subunit has some role to play in this process. Whether this is in its capacity as an enzyme of detoxification and its function is protection of cellular components during this process, or whether it has a more fundamental role to play in cell division and proliferation has yet to be established.

6.03c The role of growth hormone in the sexually differentiated induction of Yf during preneoplasia

The induction of liver cancer in the resistant hepatocyte model is markedly sex-dependent. Much faster growth of preneoplastic lesions and an earlier development of hepatocellular carcinoma is observed in male rats as compared to females (Blank *et al.*, 1984). This sex-difference in susceptibility to chemical carcinogenesis appears to be mediated via the growth hormone secretory pattern. Continuous infusion of growth hormone into male Wistar rats leads to a decline in the development of preneoplastic lesions to that observed in the female. (Blank *et al.*, 1986; 1987). Recently, Gustafsson and his co-workers have shown that sex-differences exist in the expression of c-myc and c-fos during the preneoplastic stage (Halstrom *et al.*, 1989a). Moreover, the growth hormone secretory pattern regulates the sex-specific expression of c-myc and c-fos during the early stages of chemical carcinogenesis (Halstrom *et al.*, 1989b).

The marked induction of the Yf GST during the preneoplastic process has also been reported to have a remarkable sex-dependence in favour of the male (Sato,1988). As has been discussed above, the oncogenes c-myc and c-fos are also expressed

during the early stages of this process in a sex-dependent manner. These oncogenes have also been reported as being inducible with TPA suggesting that the genes encoding these proteins also have TRE sequences contained within the 5' noncoding region (Angel *et al.*, (1989).

The pattern of regulation would appear to be very similar in certain respects between these oncogenes and the Yf GST and further suggests a common regulatory link between the genes involved in cellular proliferation, inappropriate or aberrant expression of which can lead to cancer, and the Yf GST. The significance of these findings are not clear.

6.04 Sex-related incidence of hepatocarcinoma in the mouse.

Kemp and Drinkwater (1989a) have reported on the susceptibility of six inbred strains of mice to chemically-induced hepatocellular carcinoma. Between the males of each strain a significant variation in the incidence of liver tumours was observed. Females from all strains were significantly more resistant to the effects of hepatocarcinogens, tumour frequency was much lower than in the male and no strain differences were observed when the females from different strains were compared. Furthermore, no sex differences were observed in the development of lung tumours suggesting that this difference in susceptibility was not manifest in all tissues.

Sex differences in tumour susceptibility are believed to be related to the tumour promoting ability of androgens. Neonatally castrated mice display a similar frequency of liver tumours to that seen in the female (Toy, 1981). Females administered androgens show a higher susceptibility to chemical carcinogenesis than normal females (Moore *et al.*, 1981). Tfm males lacking the androgen receptor are

also found to exhibit a feminine frequency of liver tumours (Kemp and Drinkwater, 1989b).

It is interesting to note that all changes detailed above also effect levels of the Yf subunit in a manner which correlates with the frequency of tumour development. This might simply relate to general changes in drug-metabolising enzymes which render the animal more susceptible to the effects of carcinogens. It is worth noting that in the mouse, females metabolise drugs more efficiently than in the male (MacCleod and Shapiro, 1989).

Alternatively, the expression of high levels of Yf in the male mouse, a protein which has been associated in the rat with the preneoplastic process in the liver, might be associated with this observed predisposition to liver cancer, both spontaneous and induced. An investigation into the expression of the Yf and its relationship to mouse oncogene expression might provide a very useful insight into this question.

6.05 Sex-specific expression of GST subunits in the normal mouse liver.

Sex-differences in the hepatic GST content in normal rat liver have been reported (Kaplowitz *et al.*, 1975; Hales and Neims, 1976; Lamartiniere, 1981; Igarashi *et al.*, 1985). Male rats exhibit significantly higher levels of Yb_1 as compared to the female with a concomitantly higher activity towards DCNB, approximately 2-3 -fold higher. Alpha class subunits are expressed at higher levels in the female. Thus, sex-differences exist in activity towards organic peroxides.

The mechanism underlying this dimorphic expression of GST is at present unclear. Lamartiniere (1981) investigated the role of the hypothalamic-hypophyseal-gonadal axis on the expression of rat hepatic GST. The effect of a variety of endocrine treatments on enzyme activities towards CDNB and DCNB were monitored. The results obtained suggest the pituitary has a role to play in the sexual differentiation of rat hepatic GST, but the findings are not entirely clear.

In contrast to the mouse, postpubertal castration in the rat had no effect on the high DCNB activity found in the male. Administration of exogenous androgen or oestrogen had no effect on males or gonadectomised males. DCNB activity was observed to increase in ovarectomised females treated with testosterone.

Neonatal castration of male rats resulted in a significant decrease in DCNB activity. This would suggest that the the Yb gene family is neonatally imprinted at birth, however, treatment with testosterone either in the neonatal period or in adulthood was not capable of restoring the phenotype. Again this is in contrast to the mouse where testosterone, at the appropriate dose, was able to restore, at least partially, the sex-specific expression of YfYf after castration.

Hypophysectomy was found to lead to an increase in DCNB activity in both the male and the female. Transplantation of ectopic pituitaries under the kidney capsule reversed this effect as did treatment with growth hormone. This would suggest that growth hormone is acting in a negative fashion in this system. However, lesions of the arcuate nucleus of the hypothalamus, which would also lead to a shut-down in growth hormone production and secretion, and indeed was shown to cause an decline in plasma growth hormone levels, resulted in a decline in DCNB activity. This is contradiction with earlier results, if growth hormone serves to repress hepatic

DCNB activity, then this treatment would be expected to increase activity as is the case on hypophysectomy. Hales and Neims (1976) reported that on hypophysectomy DCNB activity in the male was decreased as was the level of alpha class GST in the female. This is more in accordance with findings in other systems. Obviously further work will be required to resolve this discrepancy between these reports as they suggest different mechanisms of growth hormone action.

Interestingly, a putative clone encoding the rat Yb_1 subunit has been isolated from the rat ventral prostate (Chang *et al.*, 1987). This clone differs by two residues from that isolated and characterized by Ding *et al.* (1985) from rat liver. Whether this is a novel form of Yb_1, expressed in a tissue-specific manner remains to be established. Messenger RNA encoding this subunit was found to be repressed by androgens. The possibility raised however is that tissue-specific expression of GST might also occur at the level of regulation, in that the same factor might exert very different responses on the same gene between tissues .

6.06 Endogenous factors controlling gene expression

The glutathione S-transferases are subject to a marked tissue-specificity in their expression. This has been noted in rat, mouse and man (Hayes and Mantle, 1986; Hayes *et al.*, 1987). These differences are observed at the pre-translational level at least in the rat (Sato *et al.*, 1987). Sadly, virtually nothing is known of the endogenous factors involved in maintaining the qualitative and quantitative differences between tissues.

More information has come to light on the sex-specific control of GST. Certainly in the mouse, testosterone and growth hormone mediate these differences in expression

(Hatayama *et al.*, 1986; this thesis). In the rat, although the situation is not as clear, these hormones would also seem to play a role in this process (see section 6.05). The work presented within this thesis was primarily concerned with the regulation of the mouse hepatic Yf subunit. It was of interest to note that that the mouse Ya_1 subunit would also appear to be under the control of growth hormone, although in this case growth hormone would seem to be having a negative effect on the expression of this subunit. Further work would have to undertaken to confirm this finding.

Very little information is available on the role of other endocrine systems in the control of GST expression. While some reports are available on the effects of thyroid hormones on hepatic GST content (Arias *et al.*, 1976; Williams *et al.*, 1986; Beckett *et al.*, 1988), nothing is known of the adrenals and their effect on GST levels either in hepatic or extrahepatic tissue. It is also important to emphasize that within a tissue many cell-types exist which also display differences in GST content. In future, it will be crucial when investigating the regulation of these genes to ascertain via immunohistochemistry which cell types within the tissue are actually affected by e.g. xenobiotic treatment. Expression of GST in many individual cell types will probably be mediated via the elusive factors which govern the cellular differentiation process.

Hopefully within the next few years more attention will be paid to this vitally important, but untapped, area of research within this field, providing a better understanding of the "natural" factors which govern the complex expression of the glutathione S-transferases.

6.07 Exogenous regulation

Studies of rat PB-inducible P-450 enzymes showed that the induction of certain members of this family by PB was enhanced by hypophysectomy. Growth hormone appeared to be involved in the suppression of basal levels of these proteins, which were enhanced when the pituitary was removed, and further increased by xenobiotic treatment. This work prompted a study, detailed in chapter 5, into the effect of phenobarbital on the mouse hepatic GST content and possible modulation of this induction by the pituitary, more specifically growth hormone (Yamazoe *et al.*, 1987).

Dexamethasone, a synthetic glucocorticoid, and TCBOP, a very potent PB-like inducer in the mouse were also investigated. Both of these compounds, and phenobarbital itself, were found to cause an elevation in the mu class in both the C57BL/6 and DBA/2 strains. The constitutively expressed alpha class subunit (Ya_3) is relatively unaffected by xenobiotic treatment. TCBOP, in addition to being the most potent inducer of the mu class GST, also caused an elevation in the level of the BHA-inducible Ya subunit (Ya_1). Removal of the pituitary had a similar effect, and when both treatments were combined an additive, enhanced induction was observed. Hypophysectomy did not significantly effect the induction of the mu class GST.

Reports by other workers provide evidence to support these findings. Phenobarbital was found to increase hepatic levels of Yb-type subunits in the mouse. Interestingly, a greater effect was observed when phenobarbital was administered in the diet. (Di Simplico *et al.*, 1989). David and Nerland (1983) found DCNB activity to be induced 5-fold in mouse liver when phenobarbital was administered in the diet.

All compounds tested to date on the mouse, including the dietary additive BHA and cafestol palmitate predominantly affect the mu class GST(Di Simplico *et al.*, 1989). A lesser effect, or no effect at all, is observed with other GST classes (exception Ya_1). This is in contrast to the rat where both alpha and mu class GST members are induced in response to xenobiotic treatment. As with almost all aspects of GST research, the rat provides the best characterised model in regards to the mechanism underlying the regulation of GST by xenobiotics. This is dealt with in next section.

6.08 Xenobiotic Regulation of Rat Hepatic GST

Phenobarbital and 3-methylcholanthrene, classical inducers of two major xenobiotic regulated P-540 families, have been extensively studied with respect to their effects on rat hepatic GST. Early studies indicated that GST levels are elevated after the administration of phenobarbital and 3-methylcholanthrene (Kaplowitz *et al.*, 1975; Hayes *et al.*, 1979). The increase in protein content is also paralleled by a corresponding increase in mRNA levels. Messenger RNA encoding the Ya, Yb_1 and Yb_2 subunits are significantly elevated by treatment with these compounds. The mRNA species encoding the Yc subunit is only marginally affected. Further studies demonstrated that members of the alpha and mu classes are transcriptionally activated by xenobiotic treatment (Pickett *et al.*, 1987;1988)

Very little information is available on the underlying mechanism which mediates the effects of phenobarbital on the expression of drug-metabolising enzymes. In contrast, the regulatory pathway by which 3-methylcholanthrene and other polycyclic aromatic hydrocarbons affect the expression of specific genes has been extensively documented (for review, see Eisen *et al.*, 1983).

A cytosolic receptor, termed the Ah receptor binds avidly planar aromatic hydrocarbons such as TCDD, 3-methylcholanthrene and β-naphthoflavone. The ligand receptor complex is then translocated to the nucleus where, by some manner as yet unclear, transcriptional activation of PAH-inducible genes occurs. A diagram of the possible pathways in which P-450 and GST expression might be mediated by the Ah locus is presented in figure 6.02.

In this model, the Ah receptor binds polycylic hydrocarbons forming a ligand complex that is translocated to the nucleus and interacts with positive regulatory elements Xenobiotic Responsive Elements, (XRE) leading to the transcriptional activation of the P-540 gene and possibly GST genes. Alternatively, the receptor-ligand complex could potentially interact with another regulatory gene leading to the transcriptional activation of the regulatory gene, producing a transacting protein which could stimulate the transcription of the GST gene.

Talalay has suggested the possibility that GST substrates act as inducers of GST, by some mechanism not specified (Talalay *et al.*,1988; Spencer *et al.*, 1989). This suggests another possible variation to the model presented in figure 6.02. Transcriptional activation of PAH- inducible P-450s results in a corresponding increase in the levels of protein. This, he speculates, would also result in higher concentrations of reactive intermediates which are generated by the metabolism of P-450 substrates. These compounds are of course potential substrates for GST enzymes and, according to the model proposed by Talalay, they would also act to induce GST levels. Thus, in this model, elevation of GST levels after xenobiotic treatment are a consequence of P-450 production and metabolism rather than any direct effect of the Ah receptor ligand on GST transcriptional activation.

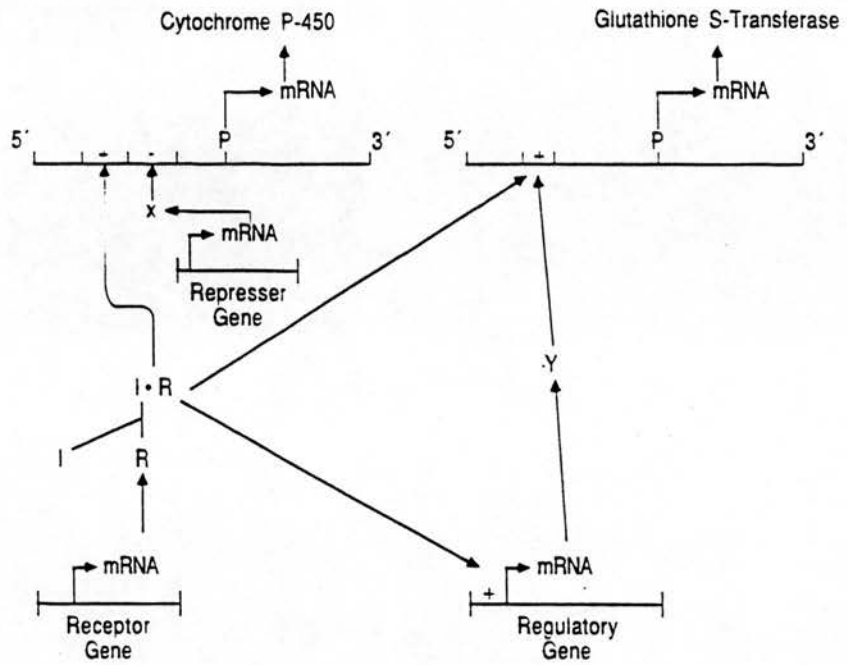


Figure 6.02 P-450 and GST regulation by polycyclic aromatic hydrocarbons

A model to account for the regulation of the glutathione S-transferase gene expression is presented along with the current model governing P-450 gene regulation by polycyclic aromatic hydrocarbons. In this model the Ah or dioxin receptor binds polycyclic aromatic hydrocarbons (I) forming a receptor ligand complex that is translocated to the nucleus and interacts with positive regulatory elements leading to the transcriptional activation of the P-450 gene and possibly the glutathione S-transferase Ya gene. Alternatively, the receptor ligand complex (I.R) could potentially interact with a regulatory gene producing a transacting protein which regulates the expression of the glutathione S-transferase Ya gene. Finally, in the P-450 system a repressor protein (X) is thought to regulate negatively the constitutive expression of the P-450 in the absence of ligand.

It has not yet been fully established which of these models is correct i.e. whether the Ah locus is acting directly to mediate the transcriptional activation of GST genes or if the effect is indirect through the activation of an another regulatory factor or P-450 metabolic products which would in turn act as both substrates for, and inducers of, GST. Whichever pathway operates in this instance, the Ah receptor is necessary for the induction of GST by polycyclic aromatic hydrocarbons (Telakowski-Hopkins *et al.*, 1988; Prochaska *et al.*, 1989).

Pickett and his co-workers, in an elegant series of experiments have investigated the 5' regulatory region involved in the induction of the rat Ya gene by polycyclic aromatic hydrocarbons. Telakowski-Hopkins *et al.*, (1988) constructed chimeric genes using various lengths of the 5' noncoding sequence of the rat Ya gene fused to the bacterial chloramphenicol acetlytransferase gene (CAT). The expression of CAT is determined by the regulatory elements contained within the 5' region. Two cis-acting regulatory elements were identified, located between 650 - 1550 bp upstream from the transcription start site. One was required for the induction of the Ya subunit by βNF and other was necessary for maximum basal levels of expression.

With respect to the PAH-inducible P-450 gene family, gene expression has been shown to be mediated via xenobiotic responsive domains, located upstream from the transcriptional start point. These xenobiotic domains have the properties of transcriptional enhancers. A consensus sequence, given below has been identified with an invarient 6 bp sequence (underlined) which is conserved between human, rat and mouse.

PAH-INDUCIBLE ELEMENT - CONSENSUS SEQUENCE

5' GNTAGCTGGG 3'

C GG

A more detailed account of the rat Ya βNF-inducible element was given by Dr C.B. Pickett at the 3rd International GST conference held in Edinburgh, 1989. The cis-acting PAH-inducible element is located 697 - 723 bp upstream of the transcriptional start site and appears to contain a unique DNA sequence which apparently is not related to the P-450 consensus sequence detailed above (Pickett *et al.*, 1989). This suggests that the Ah locus mediates the induction of P-450 and GST by xenobiotics via different routes. Obviously further studies will need to be undertaken to establish if this is indeed the case.

6.09 Regulation Of Mouse Hepatic GST by the Ah locus

Recombinant inbred strains of mice have been identified which differ in their response to polycyclic aromatic hydrocarbons such as TCDD, 3-MC and βNF. Responsive mice such as the C57BL/6 strain have been shown to have 30 to 50-fold more functional Ah receptor molecules than non-responsive strains such as the DBA/2 strain (Okey *et al.*,1979).

Phenobarbital and phenobarbital-like inducers are found to increase the hepatic GST content of both strains of mouse to similar extents (Felton *et al.*, 1980; this thesis). This is expected as phenobarbital acts via a different pathway. It is also worth noting, however, that the strains C57BL/6 and DBA/2 are also genetically distinct at

the P-450 locus encoding coumarin hydroxlyase activity, with DBA/2 having high basal activity, while C57BL/6 has a low activity. Phenobarbital is also found to produce a significantly greater response in the DBA/2 strain. This would suggest that the factors involved in conferring this difference in responsiveness do not affect the GST response to phenobarbital.

Felton *et al.* (1980) investigated the induction of mouse hepatic GST by 3-methylcholanthrene in both the C57BL/6 and DBA/2 strains. CDNB activity in the C57BL/6 strain was induced upon treatment with 3-MC or βNF, this was not observed in the DBA/2 strain. However when progeny from a cross between the two strains were investigated no association was observed between GST induction and the presence of the Ah receptor. The authors concluded that the induction of GST by 3-MC and other polycyclic aromatic hydrocarbons was not mediated via the Ah locus. Unfortunately, in this report both male and female, mature and immature mice were used interchangable. The difference in CDNB activity due to the sex-specific expression of the Yf GST was not recognised. Nor was the fact that the Yf is developmentally regulated regulated in the male (Hatayama *et al.*, 1986) taken into account. The lack of association between CDNB activity and the Ah locus is therefore by no means conclusive and indeed the data obtained from the parental strains would strongly indicate that induction of mouse hepatic GST is mediated via the Ah locus.

Further evidence for this statement has recently been provided by Daniel *et al.* (1989) who have investigated the regulatory elements 5' to a mouse genomic Ya gene in a similar manner to that described by Telakowski-Hopkins *et al.*, (1988) for the rat. As in the rat genomic Ya sequence, two cis-acting regulatory elements have been identified. One located in the sequence between the transcriptional start point and -0.2 kb was required for basal levels of expression and the other located -0.2 to 1.6

kb upstream is responsible for the inducible expression of this gene by PAH. A functional Ah receptor was also required for responsiveness to these compounds.

In contrast to the rat, where the PAH-inducible element has been reported as containing a unique sequence, the mouse 5' region was found to contain several XRE sequence motifs. Although whether these elements are actively involved in the induction of the Ya gene in response to βNF remains to be established.

Sequences required for basal expression were identified in both the rat and mouse. The element required for basal expression in the rat was found to contain a consensus HNF1 sequence (hepatocyte nuclear factor 1). This motif has been reported to mediate liver specific expression by allowing the binding of the HNF1 transacting factor (Pickett, et al. 1989). The promoter region of the mouse does not contain this sequence (Daniel *et al.*, 1989).

6.10 Regulatory differences in GST expression

Between species, the qualitative and quantitative expression of GST differs quite markedly. Tissue-specific as well as sex specific and developmental differences have been described. Differences also exist between species in their response to xenobiotic agents (Astrom *et al.*, 1987). Doubtlessly, as more information on the regulatory factors which govern expression in individual species is acquired it will also reveal the underlying mechanisms which give rise to these species differences and perhaps point to any species-specific role in which the this family of drug metabolising enzymes are possibly involved.

In this context, Telakowski-Hopkins *et al.*, (1988) found that basal levels of expression of the chimeric CAT constructs containing the the rat Ya 5' sequence with the element required for basal expression were lower when transfected into mouse cells in comparison to rat and human. Absence of a transacting factor in the mouse cell which is present in both human and rat was speculated to cause this difference in expression between the three species cell lines. It might also account for the low levels of Ya1 expression in the mouse liver. Differences between species in either the transacting factors which can regulate activation or the noncoding flanking sequences to which they bind will probably prove to be ultimately responsible for the species differences in regulation, both by endogenous factors such as testosterone and growth hormone or xenobiotics such as phenobarbital and 3-methylcholanthrene.

Although the regulatory sequences governing xenobiotic-inducibility have received most attention to date, from the work presented within this thesis and that of other workers, it is also clear that the glutathione S-transferases are also regulated by endogenous factors. In addition to searching for those sequence motifs that confer xenobiotic-inducibility, it would undoubtedly prove a valuable exercise to identify those sequences responsible for conferring responsiveness to endogenous factors.

6.11 Clinical Significance of GST Regulation

A wide assortment of chemical compounds, vastly different in structure and toxicity have been shown to inhibit chemically induced-carcinogenesis in animal models (Wattenberg,1985). It would appear that these protective agents can be neatly divided into two classes. Class I protectors, termed bifunctional inducers by Talalay or type B inducers by Wattenberg, induce both Phase I and Phase II enzyme systems

and include polycyclic aromatic hydrocarbons, polychlorinated biphenyls and phenobarbital. The second class, termed monofunctional inducers by Talalay or type A by Wattenberg, induce Phase II enzymes without causing a corresponding increase in the levels of Phase I(Wattenberg, 1985; Prochaska and Talalay, 1988). Members of this class include phenolic antioxidants, aromatic isothiocyanates, thiocarbamates. Of particular interest are the dietary additives BHA, BHT and ethoxyquin which are present in the human diet. BHA in particular is noted for its low toxicity and has been shown to protect rodents against the effects of chemical carcinogenesis in certain target organs (Wattenberg,1978). Numerous groups have reported the ability of this compound to cause a dramatic elevation in GST content both at the protein and RNA level, particularly in the liver (Benson *et al.*, 1979. McLellan and Hayes, 1989).

Wattenberg and co-workers, in an elegant series of experiments, have demonstrated a close correlation in the ability of a compound to induce GST in a target organ and the increase in protection that this compound affords (Sparnins *et al.*, 1982a). However it should be noted that treatments or events such as preneoplasia which induce GST seem to induce other Phase II enzymes. Thus a general Phase II response seems to be elicited under certain conditions which probably confers protection (Wattenberg, 1985).

Findings by Wattenberg also emphasise the importance of diet in the ability to induce basal level of GST. Mice fed a standard, crude diet exhibited a higher hepatic GST activity than mice fed a partially refined diet. Natural chemicals found in common everyday foods such as cafestol palmitate (coffee) were shown to affect GST activity in mice (Lam *et al.*, 1982; Sparnins *et al.*, 1982b). This leads to the possibility that the relatively high levels of GST found in the liver are not totally "pre-set" but

the concentration observed are in part due to the continual induction of GST by components in our everyday diet. It therefore follows that differences between individuals may not simply be due to genetic factors but also to differences in dietary intake and exposure to other environmental factors.

Compounds which can increase the cell's ability to deactivate harmful electrophiles while causing no change in the enzymes which generate these reactive species would in theory provide enhanced protection against toxicity, mutagenesis and cancer. As discussed above, some evidence has been obtained to support this theory. This information could provide a basis for possible clinical strategies in the future where the emphasis could be placed on the prevention of cancer, perhaps by changing the diet to accentuate the protective effects of the Phase II enzymes or by the development of a specific, non-toxic inducer of this system, which could be for example taken by those in high risk cancer groups (Wattenberg, 1985).

Another aspect of cancer therapy where knowledge of the regulatory controls which mediate GST levels within the cell might prove invaluable is that of cancer chemotherapy. One of the major problems encountered in chemotherapy is the phenomenon of tumour cell resistance to drug treatment regimes. This ability can either be intrinsic to the tumour type or it can be acquired by a tumour type which is initially responsive to the drug(s).

There are potentially many paths by which resistance can occur, for example, entry of the drug into cell might be impaired, efflux of the drug might be enhanced, or changes might occur in the metabolism of the drug (Harris *et al.*, 1989). In this case, changes in the GST and other Phase II systems might lead to enhanced detoxification of the drug. Indeed, the GST content of tumour cells has been found to

differ from the cell-types from which they were derived (Shea and Henner, 1987; Moscow *et al.*, 1989; Howie *et al.*, 1990). The generation of cell lines resistant to anti-cancer drugs appears to involve both qualitative and quantitative changes in GST content (Clapper *et al.*, 1987; Hall *et al.*, 1987; Wolf *et al.*, 1987). Transfection of cDNA clones encoding the GST subunit Ya was found to confer resistance to alkylating agents (Mannoharan *et al.*, 1987).

This brief summary by no means covers this very active area in GST research but simply demonstrates that changes in the expression GST subunits do occur during this process and probably contribute to the overall resistant phenotype. It must be emphasised however that the degree of resistance observed is probably due to changes in a variety of cellular components which come into contact with the drug, from the cell membrane to the nucleus. Certainly, the expression of GST in these models is aberrant, using either the normal tissue or sensitive cell line as the standard. It is therefore important to understand the normal regulatory mechanism which govern GST expression and how the tumour cell manages to evade these normal controls. In answering these questions it might be possible in the future to devise methods of preventing this from occuring. Alternatively, our increasing understanding of the many xenobiotic compounds which affect drug metabolism might enable the development, in this case of a series of compounds which could potentiate the harmful effects of the cytotoxic drug.

This section has hopefully served to show that the understanding of the regulatory mechanisms which control the expression of the glutathione S-transferases and in a wider sense the drug metabolising system is not simply an interesting academic exercise, but could also be of important clinical significance.

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